

**DOCTORAL THESIS**

**Universidad Autónoma de Madrid**

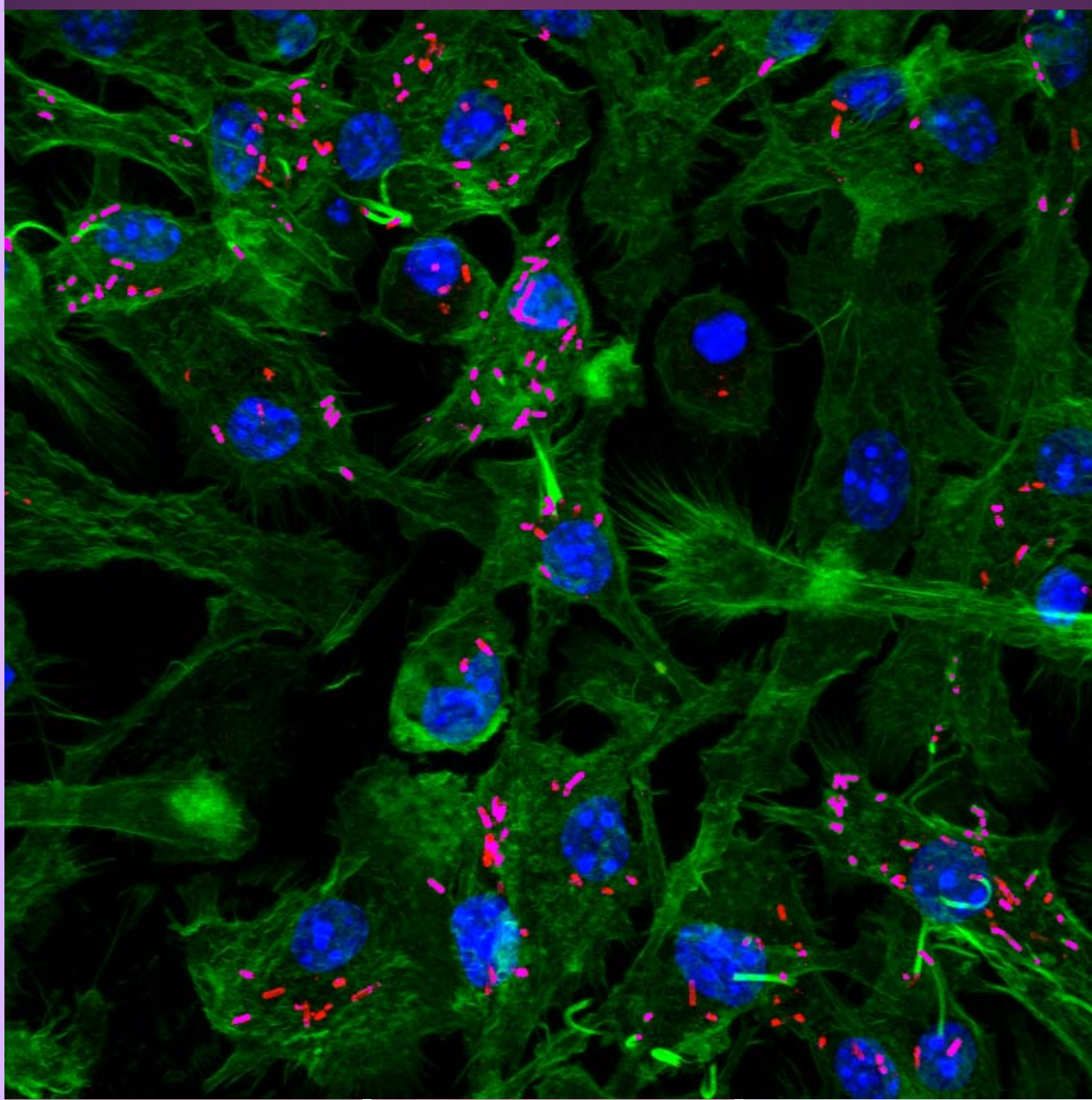
**Facultad de Medicina**

**Departamento de Bioquímica**

**Programa de Doctorado en Biociencias Moleculares**



# **Histone Deacetylase 6 at Crossroads of Infection and Innate Immunity**



**Olga Moreno Gonzalo**

**Madrid, 2018**

**Universidad Autónoma de Madrid**

Facultad de Medicina

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# **Histone Deacetylase 6 at Crossroads of Infection and Innate Immunity**

Memoria presentada por la licenciada en Biotecnología:

**Olga Moreno Gonzalo**

Para optar al título de Doctor por la Universidad Autónoma de Madrid

**Doctorado en Biociencias Moleculares**

Director de tesis:

**Dr. Francisco Sánchez-Madrid**

Doctor en Ciencias Biológicas y Catedrático de Inmunología de la  
Universidad Autónoma de Madrid

Este trabajo se realizó en el Hospital Universitario de la Princesa  
(HUP) y en el Centro Nacional de Investigaciones Cardiovasculares  
(CNIC)

Madrid, 2018

Francisco Sánchez Madrid, Doctor en Ciencias Biológicas y Catedrático de  
Inmunología de la Universidad Autónoma de Madrid,

CERTIFICA:

Que Olga Moreno Gonzalo, Licenciada en Biotecnología por la Universidad  
Francisco de Vitoria, ha realizado bajo su dirección el trabajo de investigación  
correspondiente a su Tesis Doctoral con el título:

**Histone Deacetylase 6 at Crossroads of Infection and  
Innate Immunity**

Revisado este trabajo, el que suscribe lo considera satisfactorio y autoriza su  
presentación para ser evaluado por el tribunal correspondiente.

Y para que así conste y a los efectos oportunos, firma el presente certificado en  
Madrid a 12 de marzo de 2018.

Fdo.: Prof. Francisco Sánchez-Madrid

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*A mis padres,  
a Maryan  
y a David*

*The important thing is not to stop questioning.  
Curiosity has its own reason for existing.*

*(Lo importante es no dejar de hacerse preguntas.  
La curiosidad tiene su propia razón de existir)*

*Albert Einstein  
(1879-1955)*

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# Summary

## SUMMARY

Recent evidence on Histone deacetylase 6 (HDAC6) function underlines its role as a key protein in the innate immune response to viral infection. However, whether HDAC6 regulates innate immunity during bacterial infection remains unexplored. To assess the role of HDAC6 in the regulation of defence mechanisms against intracellular bacteria, we used the *Listeria monocytogenes* (*Lm*) infection model. Here we describe that in the absence of HDAC6 Granulocyte/Macrophage colony stimulating factor-derived dendritic cells (GM-CSF DCs) display higher intracellular bacterial titres than their *wild type* (*wt*) counterparts. The impaired bacterial clearance appears to be caused by a defect in autophagy. This correlates with an increase in the accumulation of the autophagy marker p62 in *Hdac6*<sup>-/-</sup> DCs, due to a defective phagosome-lysosome fusion. We also identify higher levels of acetylated cortactin co-localizing with intracellular bacteria in *Hdac6*<sup>-/-</sup> DCs compared to *wt*, suggesting that this Post-translational modification (PTM) in cortactin could delay the phagosome-lysosome fusion.

Additionally, a lower expression of interferon-related genes and pro-inflammatory cytokines is detected in *Hdac6*<sup>-/-</sup> DCs after *Lm* infection. This observation is in accordance with lower nitrite production and inducible nitric oxide synthase (iNOS) induction during bacterial infection in *Hdac6*<sup>-/-</sup> DCs. Likewise, phosphorylation of Mitogen-activated protein kinase (MAPK) and mammalian target of rapamycin (mTOR) signalling pathways is reduced in *Hdac6*<sup>-/-</sup> DCs in response to *Lm*, suggesting an altered Toll-like receptor (TLR) signalling. A defective pro-inflammatory cytokine production in response to *Lm* and various TLR agonists is detected in both *Hdac6*<sup>-/-</sup> GM-CSF-derived and FMS-like tyrosine kinase 3 (FLT3L)-derived dendritic cells.

Interestingly, our data reveal the molecular association of HDAC6 with the TLR-adaptor protein Myeloid differentiation primary response gene 88 (MyD88), and how the absence of HDAC6 seems to diminish Nuclear factor  $\kappa$ B (NF- $\kappa$ B) induction after TLR stimuli. Moreover, *Hdac6*<sup>-/-</sup> mice display low serum levels of the inflammatory cytokine IL-6 and correspondingly an increased survival to a systemic infection with a lethal dose of *Lm*. These data underline the important function of HDAC6 in DCs, not only in the control of bacterial burden through degradation by autophagy, but also in the proper activation of TLR signalling. These results thus underscore an important regulatory role for HDAC6 in almost every step of DC functions against intracellular bacterial infection, highlighting HDAC6 as a new player of innate immune responses.



Resumen

## RESUMEN

Las evidencias recientes sobre la función de la Histona desacetilasa 6 (HDAC6) ponen de manifiesto su implicación como proteína esencial en la respuesta inmune innata durante la infección viral. Sin embargo, la función de HDAC6 en la inmunidad innata durante la infección bacteriana es desconocida. Para estudiar el papel de HDAC6 en la regulación de los mecanismos de defensa contra bacteria intracelulares, se usó el modelo de infección de *Listeria monocytogenes* (*Lm*). Aquí se describe como la ausencia de HDAC6 en las células dendríticas diferenciadas con el factor estimulador de colonias de granulocito/macrófago (GM-CSG DCs) causa un incremento en la carga de la bacteria intracelular comparado con la silvestre (*wt*). La deficiente eliminación bacteriana parece estar causada por un defecto en la autofagia. Este dato concuerda con un incremento en la acumulación del marcador de autofagia p62 en las *Hdac6*<sup>-/-</sup> DCs, que es debido a un defecto en la fusión del fagosoma con el lisosoma. También se han identificado niveles más altos de cortactina acetilada co-localizando con bacterias intracelulares en *Hdac6*<sup>-/-</sup> DCs, lo que sugiere que esta modificación post-traducciona (PTM) en la cortactina podría estar retrasando la fusión fago-lisosomal.

Además, se han identificado menores niveles de expresión de genes relacionados con interferón y citoquinas pro-inflamatorias en las *Hdac6*<sup>-/-</sup> DCs después de la infección con *Lm*. Esta observación concuerda con una menor producción de nitrito e inducción de la sintetasa de óxido nítrico inducible (iNOS) durante la infección en las *Hdac6*<sup>-/-</sup> DCs. Así mismo, las *Hdac6*<sup>-/-</sup> BMDCs muestran una menor fosforilación de las rutas de señalización de proteínas quinasas activadas por mitógenos (MAPK) y de la diana de mamíferos de rapamicina (mTOR) en respuesta a la infección por *Lm*, sugiriendo una alteración en la señalización de la ruta de receptores tipo Toll (TLR). Tanto las DCs derivadas de GM-CSF como las de la tirosina quinasa 3 de tipo FSM (FLT3L) tienen un defecto en la producción de citoquinas pro-inflamatorias en respuesta a *Lm* y a varios agonistas de TLR.

Se ha demostrado la asociación molecular de HDAC6 con la molécula adaptadora de TLR, el gen 88 de respuesta primaria a diferenciación mieloide (MyD88), y como la ausencia de HDAC6 disminuye la inducción del factor nuclear  $\kappa$ B (NF- $\kappa$ B) después del estímulo de los TLRs. Adicionalmente, los ratones *Hdac6*<sup>-/-</sup> muestran menores niveles séricos de la citoquina pro-inflamatoria IL-6, relacionado con una mayor supervivencia a la infección sistémica por la dosis letal de *Lm*. Estos datos remarcen la importante función de HDAC6 en las DCs, no solo por el control de la carga bacteriana por la degradación autofágica, sino también por la adecuada activación de la señalización de los TLRs.

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# Abbreviations

## LIST OF ABBREVIATIONS

<b>3-MA:</b> 3-MethylAdenine	<b>A3G:</b> apolipoprotein B m-RNA-editing enzyme-catalytic, polypeptide-like 3G
<b>5'pppRNA:</b> 5'triphosphate RNA	<b>CXCL5/10:</b> Cysteine-X-Cysteine motif chemokine Ligand 5/10
<b>aa:</b> amino acid	<b>CXCR1:</b> Cysteine-X-Cysteine motif chemokine Receptor 1
<b>ACK:</b> Ammonium Chloride Potassium	<b>CYLD:</b> Deubiquitinating enzyme
<b>ActA:</b> Actin Assembly-inducing protein	<b>DAPI:</b> 4',6-diamidino-2-phenylindole
<b>AHR:</b> Aryl Hydrocarbon Receptor	<b>DCs:</b> Dendritic Cells
<b>AKR1C13:</b> Aldo-keto reductase family 1 member C13 or RECON	<b>DD:</b> double deacetylase domain mutant of HDAC6 (H216A/H611A)
<b>AKT:</b> Protein Kinase B or AKT (PKB/AKT)	<b>DD1:</b> Deacetylase domain 1 of HDAC6
<b>AP-1:</b> Activator Protein-1	<b>DD2:</b> Deacetylase domain 2 of HDAC6
<b>APC:</b> Allophycocyanin	<b>DMB:</b> Dynein Motor binding domain
<b>APC:</b> Antigen presenting cell	<b>DMEM:</b> Dulbecco's Modified Eagle Medium
<b>ARP2/3 complex:</b> Actin Related Protein (subunits 2 and 3) complex	<b>DMSO:</b> Dimethyl Sulfoxide
<b>ATCC:</b> American Type Culture Collection	<b>dpi:</b> days post-infection
<b>ATG:</b> AuTophagy-related gene	<b>DPI:</b> Diphenyliodonium Chloride
<b>AU:</b> Arbitrary Units	<b>DSS:</b> Dextran Sodium Sulphate
<b>Baf:</b> Bafilomycin A1	<b><i>E. coli:</i></b> <i>Escherichia coli</i>
<b>BAHD1:</b> Bromo Adjacent Homology Domain-containing 1 protein	<b>EDTA:</b> Ethylenediaminetetraacetic acid
<b>BHI:</b> Brain Heart Infusion	<b>eGFP:</b> enhanced Green Fluorescent Protein
<b>BMDCs:</b> Bone Marrow-derived Dendritic Cells	<b>EGFR:</b> Epidermal Growth Factor Receptor
<b>BMDMs:</b> Bone Marrow-Derived Macrophages	<b>EGTA:</b> Ethylene glycol bis-( $\beta$ -aminoethyl ether)-N,N'-tetraacetic acid
<b>BNIP3L/NIX:</b> mitophagy-inducing proteins, BCL2/adenovirus E1B 19-kDa interacting protein 3-like (BNIP3L)/Nictalopin	<b>ELISA:</b> Enzyme-Linked ImmunoSorbent Assay
<b>BSA:</b> Bovine Serum Albumin	<b>ER:</b> Endoplasmic Reticulum
<b>BUZ domain:</b> Binding-of-Ubiquitin Zing finger domain of HDAC6	<b>ERK:</b> Extracellular signal-Regulated Kinase
<b>CD:</b> Cluster differentiation	<b>FC:</b> Flow Cytometry
<b>cDC:</b> conventional DC	<b>FCGR1A:</b> high affinity immunoglobulin gamma Fc receptor I or CD64
<b>c-di-AMP:</b> cyclic di-AMP	<b>FITC:</b> Fluorescein Isothiocyanate
<b>CDN:</b> Cyclic DiNucleotide	<b>FLT3L:</b> Fms-related tyrosine kinase 3 ligand
<b>CFUs:</b> Colony-Forming Units	<b>Foxp3:</b> Forkhead box P3
<b>cGAMP:</b> cyclic Guanosine monophosphate (GMP)-Adenosine MonoPhosphate (AMP)	<b>GAPDH:</b> Glyceraldehyde-3-phosphate dehydrogenase
<b>cGAS:</b> cyclic GMP-AMP synthase	<b>GM:</b> geometric mean
<b>CK2:</b> Casein Kinase II	<b>GM-CSF:</b> Granulocyte/Macrophage Colony-Stimulating Factor
<b>CMA:</b> Chaperone-Mediated Autophagy	<b>gp120:</b> envelope glycoprotein 120 of HIV
<b>CpG:</b> Cytosine-phosphate-Guanine oligodeoxynucleotide	<b>GRK2:</b> G protein-coupled Receptor Kinase 2



**GSK3 $\beta$** : Glycogen Synthase Kinase 3 beta  
**HA**: influenza HemmAgglutinin protein  
**HATs**: Histone Acetyl Transferases  
**Hda1**: yeast histone DeAcetylase 1  
**HDAC6**: Histone Deacetylase 6  
**HDAC6i**: HDAC6 inhibitors  
**Hdac6<sup>tg</sup>**: Overexpressing HDAC6 mice  
**HDACi**: Histone Deacetylase inhibitors  
**HDACs**: Histone Deacetylases  
**HIV-1**: Human Immunodeficiency Virus type-1  
**HKLM**: Heat killed *Listeria monocytogenes*  
**HKST**: Heat killed *Salmonella* Typhimurium  
**hpi**: hours post-infection  
**HRP**: Horseradish Peroxidase  
**Hsc70**: Heat Shock Cognate 70  
**HSF1**: Heat Shock Transcription Factor 1  
**HSP70/90**: Heat Shock Proteins 70/90  
**Hx**: Highly Cross-adsorbed  
**IAV**: Influenza A virus  
**IF**: ImmunoFluorescence  
**IFIT3**: IFN-Induced protein with Tetratricopeptide repeats 3  
**IFNAR-I**: IFN- $\alpha/\beta$  Receptor  
**IFN- $\alpha/\beta/\gamma$** : Interferon  $\alpha/\beta/\gamma$   
**Ig**: Immunoglobulin  
**IKK**: I $\kappa$ B Kinase  
**IL**: Interleukin  
**Ini**: Internalin  
**iNOS**: inducible Nitric Oxide Synthase  
**IP**: ImmunoPrecipitation  
**IRAK**: Interleukin-1 Receptor-Associated Kinase  
**IRF3**: Interferon Regulatory Factor 3  
**IS**: Immune Synapse  
**ISG-15**: Interferon-Stimulated Gene 15  
**JNK**: c-Jun N-terminal Kinase  
**K63/48 polyUb**: K63/48 poly-ubiquitin chains  
**kDa**: KiloDalton  
**LAMP-1/2**: Lysosome Associate Membrane Protein 1/2  
**LC3b**: Microtubule-associated protein Light-Chain 3 b  
**LFA-1**: Lymphocyte Function-associated Antigen 1

**LIR**: LC3-Intercating region of LC3  
**LLO**: Listeriolysin O  
**Lm**: *Listeria monocytogenes*  
**LntA**: *Lm* virulence factor Nuclear-Targeted protein A  
**LPS**: Lipopolysaccharide  
**Lys or K**: Lysine  
**mAb**: Monoclonal Antibody  
**MAL**: MyD88-Adapter-Like  
**MAPK**: Mitogen-Activated Protein Kinases  
**MAVS**: Mitochondrial Anti-Viral-Signalling Protein  
**M-CSF**: Macrophage Colony-Stimulating Factor  
**mDia1/2**: diaphanous-related formin 1/2  
**Mdp3**: Microtubule-associated protein 7 (MAP7) Domain-containing Protein 3  
**MEFs**: Mouse Embryonic Fibroblasts  
**MEK**: Mitogen-activated protein kinase kinase, MAP2K  
**MHC-I/II**: Major histocompatibility complex class I/II  
**miRNA**: microRNA  
**MMP2**: Matrix MetalloProtease 2  
**moDCs**: monocyte-derived Dendritic Cells  
**MOI**: Multiplicity Of Infection  
**mRNA**: messenger RNA  
**MT**: Microtubule  
**MTOC**: MT Organizing-Centre  
**mTORC1**: mammalian Target Of Rapamycin Complex 1  
**MVP**: Major Vault Protein  
**Mx1**: Myxovirus resistance 1  
**MyD88**: Myeloid Differentiation primary response Gene 88  
**NADH**: reduced Nicotinamide Adenine Dinucleotide  
**NADPH oxidase/NOX2**: Nicotinamide Adenine Dinucleotide Phosphate oxidase  
**NBR1**: Neighbor of BRCA1 gene 1 protein  
**NDP52**: nuclear dot protein 52 kDa  
**NEMO**: NF- $\kappa$ B Essential MOdulator  
**NES**: Nuclear Import Signal  
**NF $\kappa$ B**: Nuclear Factor kappa B

**NH<sub>4</sub>Cl:** ammonium chloride  
**NI:** Non-Infected  
**NLR:** NOD-like Receptor  
**NLRP3:** NLR family Pyrin domain-containing 3  
**NLS:** Nuclear Localization Signal  
**NO:** Nitric Oxide  
**NOD:** Nucleotide-binding Oligomerization Domain  
**NOS:** Nitric Oxide Species  
**OVA:** Ovalbumin  
**P97/VCP:** p97/Valosin-Containing Protein  
**pAb:** Polyclonal Antibody  
**Pam2GSK4:** palmitoyl-2 cysteine-serine-(lysine)<sub>4</sub>  
**Pam3GSK4:** palmitoyl-3 cysteine-serine-(lysine)<sub>4</sub>  
**PAMPs:** Pathogen-Associated Molecular Patterns  
**PBMC:** Peripheral Blood Mononuclear Cells  
**PBS:** Phosphate Buffered Saline  
**PCR:** Polymerase Chain Reaction  
**PE:** phosphatidylethanolamine  
**PE:** Phycoerythrin  
**PeCy7:** PE-Cy7  
**PerCP:** Peridinin-chlorophyll Protein Complex  
**PI3K:** Phosphatidylinositol 3-Kinase  
**PKC:** Protein Kinase C  
**PLC:** Phospholipase C  
**Poly(I:C):** Poly(inosinic:cytidylic acid)  
**PrxI/II:** Peroxiredoxin I/II  
**PTMs:** Post-Translational Modifications  
**PVDF:** Polymer of Vinylidene Fluoride  
**RAF:** Mitogen-activated protein kinase kinase Kinase, MAP3K  
**Rag1<sup>-/-</sup>:** Recombination-activating gene 1 mice  
**RanBPM:** Ran-Binding Protein M  
**RAS:** Rapidly Accelerated Fibrosarcoma  
**RD:** Repressor domain of RIG-I  
**RFP:** Red Fluorescent Protein  
**RIG-I:** Retinoic acid Inducible Gene-I  
**RNS:** Reactive Nitrogen Species  
**ROS:** Reactive Oxygen Species  
**Rpd3:** yeast Reduced potassium dependency-3

**RPMI:** Roswell Park Memorial Institute-1640  
**RT-qPCR:** Reverse Transcription-quantitative Polymerase Chain Reaction  
***S. aureus:*** *Staphylococcus aureus*  
***S. Typhimurium:*** *Salmonella enterica* serovar Typhimurium  
**S:** Supernatant  
**S+P:** Supernatant + Pellet  
**SCID:** Severe Combined ImmunoDeficient mice  
**SCV:** *Salmonella*-Containing Vacuole  
**SE14:** cytoplasmic anchoring motif of HDCA6  
**SEAP:** Secreted Embryonic Alkaline Phosphatase  
**SEM:** Standard Deviation of the Mean  
**Ser:** Serine  
**SeV:** Sendai Virus  
**shRNA:** small hairpin RNA  
**Sir2:** yeast silent information regulator 2  
**Sirt:** Sirtuin  
**SLAPs:** Spacious *Lm*-containing Phagosome  
**SQSTM1/p62:** Sequestosome 1/p62  
**STING:** STimulator of INterferon Genes  
**TAB1/2/3:** TAK1-Binding Proteins 1/2/3  
**TAK1/2/4:** TGF- $\beta$  Activated Kinase 1/2/4  
**Tat:** Transactivation protein of HIV-1  
**TBS:** Tris-Buffered Saline  
**TCR:** T Cell Receptor  
**TEMs:** Thioglycollate-Elicited Macrophages  
**TF:** Transcription Factor  
**TG:** Thioglycollate  
**TGF- $\beta$ :** Transforming Growth Factor- $\beta$   
**Thr:** Threonine  
**Tip-DC:** TNF/Inducible NO synthase-Producing DC  
**TIRAP:** Toll/Interleukin-1 Receptor domain containing Adaptor Protein  
**TLR:** Toll-Like Receptor  
**TNF- $\alpha$ :** Tumor Necrosis Factor- $\alpha$   
**TRAF6:** TNF receptor-associated factor 6  
**Treg:** Regulatory T cell  
**TRIF:** TIR(Toll/interleukin-1 receptor)-domain-containing adapter-inducing IFN- $\beta$   
**TSA:** Tubastatin A

**TTP/p25:** Tubulin Polymerization Promoting protein 1/p25

**Ub:** Ubiquitin

**UBA:** Ubiquitin-Associated Domain

**UBD:** Ubiquitin-Binding Domain

**UPEC:** Uropathogenic *Escherichia coli*

**UPR:** Unfolded Protein Response

**UPS:** Ubiquitin-Proteasome System

**Vif:** Viral Infectivity Factor

**VSV:** Vesicular Stomatitis Virus

**WASP:** Wiskott Aldrich Syndrome Protein

**WB:** Western Blot

**wt:** wild-type

**Ywhaz:** (tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein,  $\zeta$ )

**$\beta$ 2-M:**  $\beta$ 2-Microglobulin

# Introduction

## 1. INTRODUCTION

The immune response is carried out by a highly specialized system and is constituted by cells and molecules that work in a coordinated and cooperative manner to react against pathogens and external threats to protect the body. The “innate immunity”, constitutes the first line of defence against pathogens, acting very fast to counteract pathogens during the earliest phase of infection. Mechanisms involved in the recognition of microbes or damaged tissues and the subsequent reaction are repeated patterns common to groups of related pathogens. On the other hand, the “adaptive immune response” is highly effective and specific to fight against particular microbes. The most important hallmark of adaptive immunity is “immunological memory”, essential to respond in a faster way and to generate a pool of memory cells that protect the body against reinfections. Innate and adaptive immune responses constitute a very precise integrated system of defence. In this regard, soluble factors delivered by innate immune responses to pathogens influence in the function of adaptive immune responses. Conversely, adaptive immune responses can focus the activity of innate immunity to be more effective to combat microbe insults.

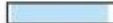







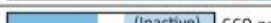



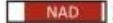




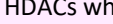
The interest of this work is focused in the understanding of immunological mechanisms involved in the role played by HDAC6 in host cell defence against infection by the intracellular bacterium *Listeria monocytogenes*.

### 1. Histone deacetylase 6 (HDAC6)

#### 1.1. Histone Deacetylase family (HDACs)

Histone deacetylases (HDACs) belong to a protein family with a mainly nuclear localization, which play an important role in the regulation of genetic expression together with other histone modifier proteins such as acetyltransferases (HATs), methylases or methyltransferases [118,51]. Histone acetylation (mediated by HATs) is a versatile process that relax the chromatin structure, increasing gene transcription, whereas histone deacetylation, (carried out by HDACs), compacts the chromatin structure, inducing genetic silencing. HDAC family is composed by proteins that at least contain one deacetylase catalytic domain responsible for the removal of acetyl groups from target proteins [51]. There are 18 mammalian HDACs that are classified into four main groups according to their homology to yeast HDACs (Figure 1.1). Class I HDACs are orthologues of yeast reduced potassium dependency-3 (Rpd3) and comprise HDAC1, HDAC2, HDAC3 and HDAC8, which remain mainly localized in the nucleus. Class II HDACs are related to yeast Hda1 and according to their sequence homology and domain structure, are subdivided into class IIa (HDAC4, HDAC5, HDAC7 and HDAC9) and class IIb (HDAC6 and HDAC10) [264]. Class III HDACs belong to the family of deacetylases called silent information regulator 2-like (Sir2) and

include seven sirtuins (Sirt-1 to 7). The class IV HDAC group is formed by HDAC11, which shares conserved catalytic core domains with Classes I and II HDACs, but shows a different structure [21]. HDAC3, Class IIa and IIb HDACs are able to interchange their cellular localization between nucleus and cytoplasm depending on several cellular signals. However, Sirt-1, 6 and 7 are mainly found in the nucleus, while Sirt-3, 4 and 5 are localized at mitochondria.

Class	Isoform	Yeast counterpart	Size	Co-factor	Location	Expression	Catalytic domain
(I)	HDAC1	RPD3	58	Zn <sup>2+</sup>	N	Ubiquitous	 482 aa
	HDAC2		59		N		 488 aa
	HDAC3		50		N, C		 428 aa
	HDAC8		44		N		 377 aa
(IIa)	HDAC4	HDA1	120	Zn <sup>2+</sup>	N, C	Specific	 1084 aa
	HDAC5		130		N, C		 1122 aa
	HDAC7		110		N, C		 952 aa
	HDAC9		160		N, C		 1011 aa
(IIb)	HDAC6	HDA2	160	Zn <sup>2+</sup>	N, C	Specific	 1215 aa
	HDAC10		70		N, C		 (Inactive) 669 aa
(III)	SIRT1	Sir2	120	NAD <sup>+</sup>	N	Variable	 NAD 747 aa
	SIRT2		45		C		 NAD 389 aa
	SIRT3		28		M		 NAD 399 aa
	SIRT4		35		M		 NAD 314 aa
	SIRT5		36		M		 NAD 310 aa
	SIRT6		39		N		 NAD 355 aa
	SIRT7		48		N		 NAD 400 aa
(IV)	HDAC11	RPD3/HDA1	39	Zn <sup>2+</sup>	N	Ubiquitous	 347 aa

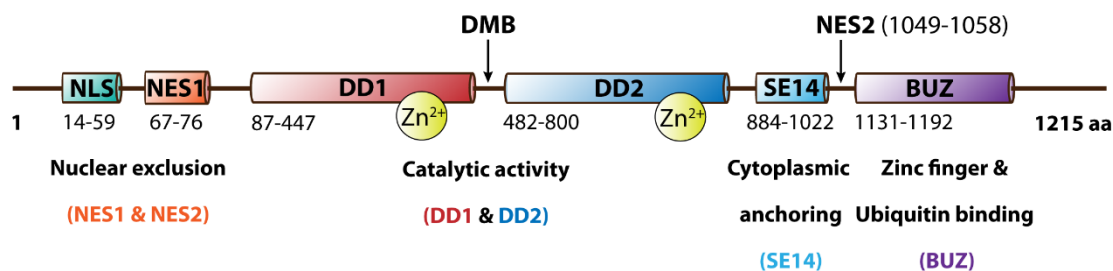
**Figure 1.1. HDACs family.** Classification of the 18 mammalian HDACs which are classified into four groups based on sequence similarity to the catalytic domain of yeast prototypes. Class I HDACs include the yeast Rpd3 homologs, HDAC 1, 2, 3 and 8. Class II HDACs encompass the yeast HDAC1/2 homologs, HDAC 4, 5, 6, 7, 9 and 10. They are further subclassified as IIa (HDAC 4, 5, 7 and 9) and IIb (HDAC 6 and 10). Class III HDACs (namely sirtuins) are composed by the yeast Sir2 orthologs SIRT 1, 2, 3, 4, 5, 6 and 7. HDAC11 belongs to the Class V HDACs, which has sequence similarity to both Rpd3 and Hda1. The subcellular localization is indicated as N: nuclear, M: mitochondrial and C: cytosolic. Sizes correspond to protein molecular weight and are expressed in kDa. From Shirakawa *et al* 2013 [223].

The common characteristic of HDACs is that they use zinc (Zn<sup>2+</sup>) like enzymatic co-factors, belonging to the zinc-depending deacetylases groups [21]. This feature distinguishes them from sirtuins or NADH-depending deacetylases [213,246]. HDACs are ubiquitous and pleiotropic proteins that can be found in all cell types, although Class II HDACs are enriched in specific tissues. All of them are expressed in all mammalian cells and are distributed in different compartments such as nucleus, cytosol and mitochondria [51]. HDACs controls gene expression and are involved in many biological processes such as cell growth, proliferation and differentiation, cytotoxicity and apoptosis [51].

## 1.2. HDAC6 Structure

HDAC6 was the first characterized HDAC to be in the cytoplasm in an active form [91]. HDAC6 has a molecular weight of 130 KDa. This protein has two functional deacetylase domains (DD1 and DD2),

which are involved in the removal of acetyl groups from target proteins such as tubulin, cortactin and Heat Shock Protein 90 (HSP90) [91,268,269,273] (**Figure 1.2**). Although both domains are functional, DD1 has a weak enzymatic activity and shows substrate specificity for C-terminal acetyl lysine residues, whereas DD2 displays broad substrate spectrum [78,156]. It also possesses the SE14 domain, built with 8 consecutive repeats of a tetradecapeptide, essential for its cytosolic retention and for interaction with Tau [20]. Moreover, its N terminal nuclear export signal (NES) is stronger than its nuclear localization signal (NLS), leading to a predominant presence of HDAC6 in the cytosol. Both signals control the cytoplasmic-nuclear shuttling process of HDAC6.



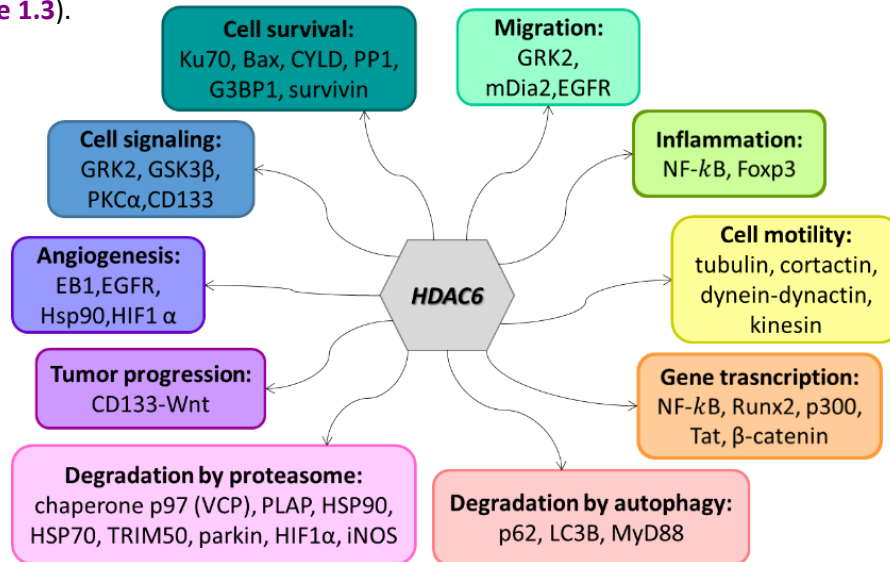
**Figure 1.2. HDAC6 structure.** HDAC6 protein is composed by the following domains. **NLS**: Nuclear localization signal, **NES 1 and 2**: Nuclear export signals 1 and 2, **DD1** and **DD2**: functional deacetylase domains 1 and 2, **DMB**: dynein motor binding domain, **SE14**: cytoplasmic anchoring motifs, **BUZ**: Binder of Ubiquitin Zinc finger domain. The corresponding amino acid (aa) sequence is showed below each domain by a range of numbers. Adapted from Li *et al* 2013 and Ageta-Ishihara *et al* 2013 [134,2].

To carry out its nuclear function, HDACs deacetylate histones modifying the packaging of the genetic material, however, HDAC6 also has a C-terminal zinc-finger ubiquitin-binding protein domain (ZnF-UBP) namely BUZ domain, that mediates the direct binding to DNA, regulating gene transcription. Besides, the ZnF-UBP domain is composed by a conserved cysteines and histidines repeat, also present in a group of proteins called ubiquitin-specific proteases, which allows HDAC6 to interact with mono- and poly-ubiquitinated chains, present in ubiquitinated proteins and in ubiquitin-interacting proteins [183,179,180]. This feature highlights the wide magnitude of the HDAC6 interactome, where a large variety of molecules can bind to HDAC6 dependently or independently of its catalytic function [244]. For this reason, HDAC6 is considered a crucial protein to link two cell signalling systems, mediated by acetylation and ubiquitination [244,31,216].

### 1.3. General functions of HDAC6

Due to the predominantly cytosolic localization of HDAC6, this enzyme is able to regulate several important biological processes such as immune response, lymphocyte activation, immune synapse organization, cell adhesion, migration, proliferation, survival, and differentiation through both deacetylase- and ubiquitin-dependent mechanisms [244,134,31,216]. HDAC6 is also involved in various pathological disorders, including autoimmunity and inflammation (autoimmune and Dextran

sodium sulphate (DSS)-induced colitis, autoimmune arthritis, and encephalomyelitis) viral infections (Human Immunodeficiency Virus (HIV), influenza A virus (IAV), Sendai virus (SeV)), neurodegenerative diseases (Parkinson and Alzheimer) and tumour progression and metastasis (solid and haematological tumours) [75]. The role of HDAC6 in relevant physiological processes is summarized in the following chart (Figure 1.3).



**Figure 1.3. Functions of HDAC6 in physiological processes.** Cellular functions are indicated in boxes, where HDAC6-interacting proteins implicated in particular cellular functions are listed.

### 1.3.1. Deacetylase-dependent functions

The first characterized HDAC6 substrate was tubulin, which is acetylated at lysine 40 (Lys<sup>40</sup>) [91,184] [151]. HDAC6 enzymatic inhibition or downregulation results in tubulin acetylation, which enhances the stability of microtubule (MT) network and the binding of the two principal molecular motors of the cell, kinesin-1 and dynein, responsible for the anterograde and retrograde transport, respectively [7,115,272]. HDAC6 also binds to the dynein-activator complex, called dynactin. HDAC6 function is necessary to transport of misfolded protein aggregates (aggresomes) to proteasomes or lysosomes for degradation, avoiding cell toxicity [115,272,107,98,32].

One of the best characterized HDAC6 substrates is cortactin, a filamentous (F)-actin binding protein, which promotes actin polymerization and microfilament branching. Acetylated cortactin has diminished F-actin binding, influencing actin-mediated cellular processes such as endocytosis, migration, chemotaxis, adhesion or intracellular transport [268,140]. Besides, HDAC6 is able to regulate in a coordinate manner the intersection between microtubule cytoskeleton and actin filaments by the interaction with mediator proteins called formin homology proteins, mDia1 and mDia2 [56,19]. Other mediator proteins are septins, which are cytoskeletal guanosine triphosphatase proteins involved in providing a physical scaffold for HDAC6 to achieve MT deacetylation [2]. For these reasons, HDAC6 is considered as a crucial regulator of cell motility, able to connect both actin and



tubulin cytoskeletons. In fact, HDAC6 deregulation has been described in many tumours due to its implication in cytoskeleton, not only in cell progression, but also in angiogenesis and metastasis [91,184,3,76,103,136].

HDAC6 deacetylates HSP90 changing its activity, which is implicated in the regulation of many fundamental cell processes. HSP90 acetylation at Lys<sup>294</sup> favours its dissociation from its co-chaperone p23, promoting the instability of many HSP90-interacting proteins involved in cell growth and survival [202,110,13,116]. New HDAC6 substrates have been characterized in last years and are summarized in

**Table 1.1.**

**Table 1.1. List of HDAC6 substrates.** The table shows new non-histone HDAC6 client proteins, lysine residue susceptible of acetylation, related function and references.

Substrates	Deacetylated residues	Related function	References
<b>α-tubulin</b>	K <sup>40</sup>	IS formation, virus infection, cell migration and chemotaxis	[91,151,220,243,36]
<b>Cortactin</b>	K <sup>87/124/161/189/198/235/272/309/319</sup>	Regulation of actin filaments, cell motility, fusion of vesicles	[268,127]
<b>HSP-90</b>	K <sup>294</sup>	kinase activation and macropinocytosis	[116,110]
<b>Tat</b>	K <sup>48</sup>	Suppression of HIV transactivation	[92]
<b>RIG-I</b>	K <sup>858</sup> and K <sup>909</sup>	Viral RNA recognition	[49,137]
<b>Ku70</b>	K <sup>539/542</sup>	Suppression of apoptosis	[232]
<b>β-catenin</b>	K <sup>49</sup>	Gene transcription activation	[271,135,145]
<b>Peroxioredoxin-1 and 2</b>	PrxI (K <sup>197</sup> ) PrxII (K <sup>196</sup> )	Redox regulation	[187]
<b>Survivin</b>	K <sup>129</sup>	Anti-apoptotic function	[207]
<b>MYH9</b>	-	Actin-binding regulation	[267]
<b>HSC70</b>	-	Protein folding	[267]
<b>DNAJA1</b>	-	Protein folding	[267]
<b>HSPA5</b>	K <sup>447</sup>	Suppression of breast cancer metastasis	[45]
<b>HMG2</b>	K <sup>2</sup>	Gene transcription and breast cancer growth	[153]
<b>Sam68</b>	-	Alternative splicing	[167]
<b>14-3-3ζ</b>	K <sup>49/120</sup>	Suppression of growth and survival signalling	[159]
<b>Tau</b>	-	Neurodegeneration	[39,155,133]
<b>Microtubule-associated protein 1 light-chain 3 (LC3b)</b>	-	Autophagy	[138]

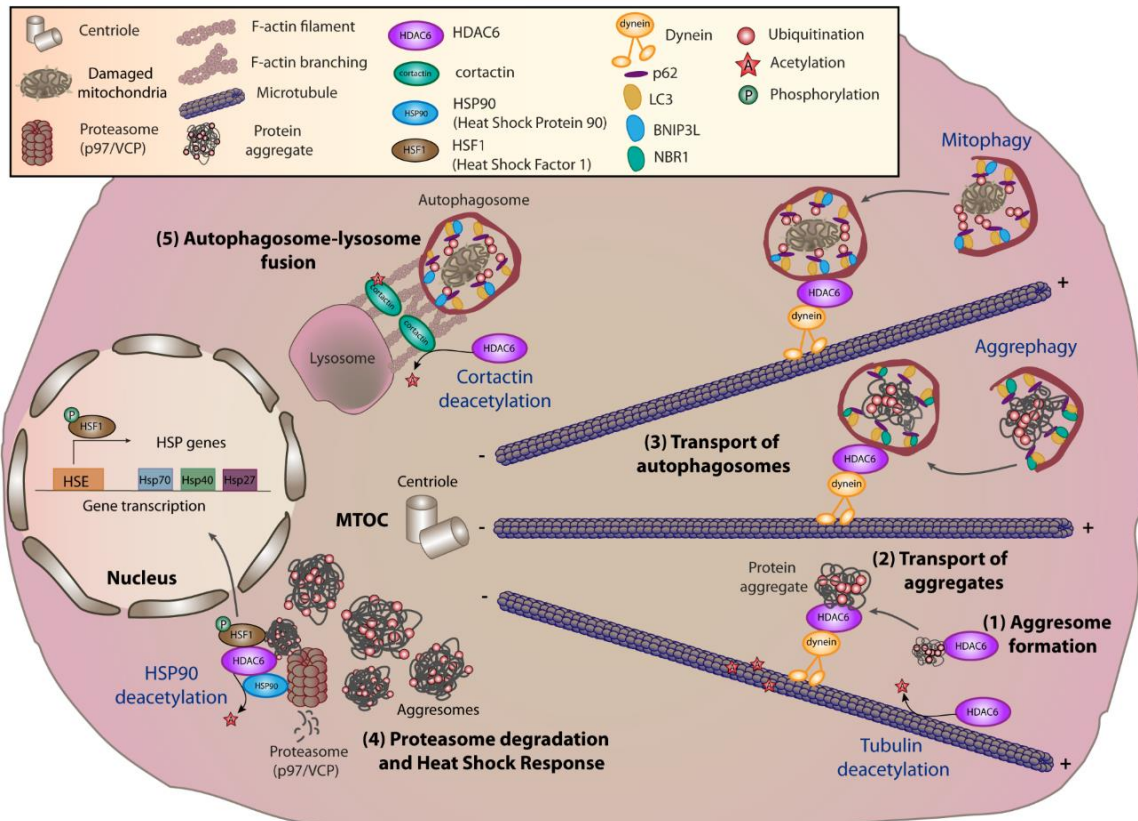
### 1.3.2. Ubiquitin-dependent functions

Through its BUZ domain, HDAC6 interacts with mono- and poly-ubiquitin chains, with a preferential binding for K63-linked ubiquitin chains [179,180]. For this reason, this protein participates in the formation of aggresomes, which transiently store toxic misfolded proteins (ubiquitinated protein aggregates). Besides, HDAC6 participates in the retrograde transport of aggregates along MT to be degraded by the primary quality control mechanism of the cell, the Ubiquitin-Proteasome System (UPS) [98,256,30,32]. HDAC6 also mediates the stability of ubiquitinated aggregates through its molecular association with the USP chaperone p97/valosin-containing protein (p97/VCP) and HSP90 [32,58]. Ubiquitinated protein aggregates are subsequently disrupted by p97/VCP machinery from

HDAC6 to deliver them to the proteasome for degradation, which promotes HDAC6 recycling [29]. In some situations, proteasomes are disrupted or overloaded, and aggresomes can be cleared by autophagy in a specific process called aggrephagy [98,58,186]. In this regard, HDAC6 is responsible for the dynein-mediated centripetal transport through MT of altered organelles and protein aggregates, concentrating them in perinuclear regions near the Microtubule Organizing Center (MTOC), where lysosomes are usually located [259,112,99]. Tubulin acetylation at Lys<sup>40</sup> increases the recruitment and mobility of dynein through MT, enhancing the transport [59,203]. Therefore, HDAC6 has an important role in ubiquitin-selective quality-control autophagy, controlling the actin and tubulin networks [127]. Selective autophagy exclusively eliminates specific cellular components, as organelles like mitochondria (mitophagy) or unfolded protein aggregates (aggrephagy) [127,268,129,74,231]. Although HDAC6 is not required for autophagy induction, its deacetylase activity over cortactin is involved in autophagosome maturation. Deacetylation of cortactin induces the local assembly of actin microfilaments necessary for autophagosome fusion with the lysosome [127]. However, this is not dispensable for starvation-induced autophagy. SQSTM1/p62 interacts with HDAC6 and recruits F-actin filaments near HDAC6, promoting their deacetylation and the subsequent autophagosome-lysosome fusion [262]. Interestingly, the ubiquitin-like interferon (IFN)-stimulated gene 15 (ISG15) is able to interact with the BUZ domain of HDAC6 due to the structural similarity between K63-polyUb and ISG15 post-translational modifications (PTMs), labelling proteins for autophagic degradation [166].

HDAC6 forms a complex with HSP90, p97/VCP and heat shock transcription factor 1 (HSF1), which is disrupted in the presence of misfolded proteins due to the binding of HDAC6 to ubiquitinated aggregates. Available HSF1 activates the heat shock response and aggregates are degraded in the proteasome, highlighting the HDAC6 ability to sense cellular stress [32,107]. The roles of HDAC6 in aggresome formation, aggrephagy, autophagy, mitophagy and Heat Shock response are schemmatically illustrated in **Figure 1.4**.

On the other hand, the lack of HDAC6 impairs mitofusin 1 deacetylation, which blocks the mitochondrial fusion induced by glucose deprivation, causing an excess of reactive oxygen species (ROS) responsible of cellular oxidative damage [126,11]. It has been reported other role of HDAC6 in the mitochondria by its interaction with parkin and HSP70, regulating the homeostasis of this organelle through the control of mitophagy [128,104]. Hence, HDAC6 is an essential player of cell homeostasis because of its regulation not only of the cross-talk between the two main cellular degradation systems, the autophagy and the proteasome, but also of mitochondrial metabolic balance [98,57,186,72,104].



**Figure 1.4. HDAC6 controls the principal clearance mechanisms of the cell.** HDAC6 is involved in the regulation of aggresomes, proteasomes, Heat Shock response (HSR), and selective autophagy through the control of actin and tubulin networks and HSP90. **(1)** HDAC6 BUZ domain binds ubiquitinated protein aggregates and promotes aggresome formation to avoid cell toxicity. **(2)** HDAC6 is able to transport aggresomes through the MT due to its binding to the molecular motor dynein. Aggresomes are concentrated near the MTOC, where proteasomes are located. **(3)** HDAC6 binds to autophagosome-contained ubiquitinated substrates (protein aggregates and damaged mitochondria) and is implicated in their dynein-mediated transport to MTOC areas, where lysosomes are accumulated. Autophagy receptors p62 and NBR1 recognize protein aggregates and BNIP3L/NIX receptors detect damaged mitochondria by their UBA domains, whereas their LIR domains bind to the membrane autophagosome marker LC3. Acetylation of MT enhances dynein transport. **(4)** HDAC6 transfers aggresomes to the proteasome to be degraded and deacetylates HSP90 to start an HSR. Phosphorylation of HSF1 leads its nuclear import to initiate the transcription of HSP genes (HSP70, 40 and 27) **(5)** HDAC6 recruits cortactin to ubiquitinated substrates, whereas p62 assembles F-actin. HDAC6 deacetylates cortactin, promoting actin filament polymerization and branching and the subsequent autophagosome-lysosome fusion. Cortactin acetylation impairs this fusion. Adapted from Banreti *et al* 2013 and Richter-Landsberg *et al* 2013 [16,206].

## 1.4. Regulation of HDAC6

There is growing evidence regarding the regulation of the HDAC6 activity that encompasses post-translational modifications (PTMs), such as phosphorylation and acetylation, and RNA silencing by microRNAs. These mechanisms are able to control the levels of both cytoplasmic and nuclear HDAC6.

### 1.4.1. Phosphorylation

Phosphorylation has been broadly observed to improve the deacetylase activity of HDAC6. For instance, phosphorylation at serine 22 (Ser<sup>22</sup>) of HDAC6 by the glycogen synthase kinase 3 (GSK3 $\beta$ )

enhances its enzymatic activity, regulating mitochondrial transport in hippocampal neurons [47]. Similar results have been detected at Ser<sup>1035</sup> of HDAC6 by the activation of epidermal growth factor receptor (EGFR)-RAS-RAF-MEK-ERK signalling axis *in vivo*, whereas ERK-1 is able to phosphorylate HDAC6 at threonine 1031 (Thr<sup>1031</sup>) and Ser<sup>1035</sup> for the regulation of cell motility *in vitro* [254]. Casein kinase II (CK2) phosphorylates HDAC6 at Ser<sup>458</sup>, enhancing its activity during the formation and the clearance of aggresomes [251]. HDAC6 is also phosphorylated at unknown residues by G protein-coupled receptor kinase 2 (GRK2) [119], Aurora A kinase [196], and protein kinase C isoform  $\zeta$  (PKC $\zeta$ ) [61]. Moreover, SeV entry activates PKC $\alpha$  activity, which phosphorylates HDAC6, promoting the deacetylation and nuclear translocation of  $\beta$ -catenin to induce antiviral response [271].

#### 1.4.2. Acetylation

HDAC6 can be modified by acetylation, affecting its deacetylase activity. Currently, it remains unknown whether HDAC6 is able to acetylate itself. Acetyltransferase p300 is responsible for HDAC6 acetylation at Lys<sup>16</sup>, impairing its tubulin deacetylation and Sp1 transcriptional activities [84]. Acetylation of HDAC6 changes its subcellular localization because this PTM breaks the interaction between HDAC6 and importin- $\alpha$ , promoting the cytosolic retention of HDAC6 and the subsequent inability to deacetylate histones [141].

#### 1.4.3. Regulators

It has been reported that several regulators interact with HDAC6 to directly inhibit its deacetylase activity. For example, tubulin polymerization-promoting protein-1 (TPPP/p25) binds to HDAC6, inhibiting its deacetylase activity on tubulin and  $\beta$ -catenin [238,215]. Paxillin also inhibits HDAC6 tubulin deacetylation, regulating Golgi structure and polarized migration [54]. The deubiquitinating enzyme CYLD negatively regulates HDAC6 activity on tubulin, which affects cell-cycle progression [253]. However, inactivation of HDAC6 by CYLD promotes ciliogenesis [265]. Mdp3 blocks HDAC6 function, promoting the acetylation of tubulin and the subsequent stabilization of microtubules [235]. The microtubule-associated protein tau inhibits HDAC6 deacetylase function [190]. Ran-binding protein M (RanBPM) interacts with HDAC6, impairing its deacetylase function, which favours aggresome formation [212]. On the contrary, absence of p62 causes an enhanced deacetylase activity of HDAC6 on tubulin and cortactin, necessary for the engulfment of aggresomes into autophagosomes [262].

#### 1.4.4. microRNA

Numerous microRNAs regulate HDAC6 expression and function, such as miRNA-22 [90,147], miRNA-26a [130], miRNA-221 [9], miRNA-433 [224,250,147] and miRNA-548m [143].

## 1.5. HDAC6 functions in the immune system

### 1.5.1. Role of HDAC6 in adaptive immunity

The regulation of tubulin cytoskeleton by HDAC6 is important in the cognate immune synapse (IS) between a T helper cell and an antigen presenting cell (APC) [220]. Acetylated microtubules are concentrated at the T cell contact area with APCs, surrounding CD3 and lymphocyte function-associated antigen 1 (LFA-1) clusters. After antigen-specific engagement of T cell receptor (TCR), a transient deacetylation of microtubules is induced at early times, followed by their subsequent acetylation at late times. Early deacetylation is carried out by HDAC6, which specifically translocates and concentrates to APC-T cell contact sites. Moreover, overexpression of HDAC6 disorganizes CD3 and LFA-1 clusters, translocation of microtubule organizing centre (MTOC), and IL-2 secretion, all necessary for a proper IS. However, overexpression of HDAC6-DD mutant does not exert the same effect and Trichostatin A (TSA) treatment reverts the effect, indicating the enzymatic contribution of HDAC6 during IS formation.

HDAC6 is also important for the chemotactic migration of the lymphocyte [36]. There is dynamic subcellular location of HDAC6 in the leading edge of lamellipodium and in the rear edge of uropod on migrating T cells. HDAC6 overexpression increases chemokine-mediated T cell migration and transendothelial migration under shear flow, whereas HDAC6 silencing diminishes T cell chemotactic capacity. The use of specific chemical inhibitors of HDAC6 and enzymatic activity mutants reveals that HDAC6 regulation of lymphocyte chemotactic migration is not mediated by its catalytic activity, but by an scaffold role.

HDAC6 plays a role in cytotoxic CD8<sup>+</sup> T lymphocytes during DNA virus infection [176]. *In vivo* immunization studies reveal a defective cytotoxic response in HDAC6 *knockout* mice. CD8<sup>+</sup> T lymphocyte adoptive transfer into Recombination-activating gene 1 (*Rag1*<sup>-/-</sup>) recipient mice demonstrates the specific impairment of cytotoxic response against vaccinia virus infection. The altered dynamics of lytic granules of HDAC6 deficient CD8<sup>+</sup> T lymphocytes is due to the lack of binding of HDAC6 to kinesin-1-dynactin, involved not only in the altered terminal transport of these granules to the synapse but also in their defective exocytosis [176].

HDAC6 exerts a role in the adaptive response of CD4<sup>+</sup> T cells in different autoimmune and inflammatory disorders such as colitis and cardiac-allograft rejection by the enhanced transcription of Foxp3 [52]. HDAC6 deficient CD4<sup>+</sup> T cells express higher levels of the transcription factor Foxp3, deregulating the balance between pro- and anti- inflammation in favour of regulatory T cells, promoting a protective role during DSS-induced and autoimmune models of colitis and fully MHC-incompatible cardiac allograft rejection [52]. Accordingly, mice treated with HDACi show acetylated

Foxp3, which increases its DNA-binding capacity, enhancing Treg differentiation [18]. HDAC9-deficient mice display HSP70 acetylation, which prevents its degradation by proteasome and results in its accumulation, enhancing HSP70-Foxp3 interaction that stabilizes Foxp3 [53]. Both observations could be also extended to HDAC6-deficient mice. Interestingly, HSP90 acetylation impairs its binding to Aryl Hydrocarbon Receptor (AHR), which affects the AHR ability to recognize ligands and the subsequent activation of AHR signalling [110]. Activation of AHR signalling pathways, enhances *in vitro* Th17 polarization [62]. Hence, Th17 response might be decreased in HDAC6 absence.

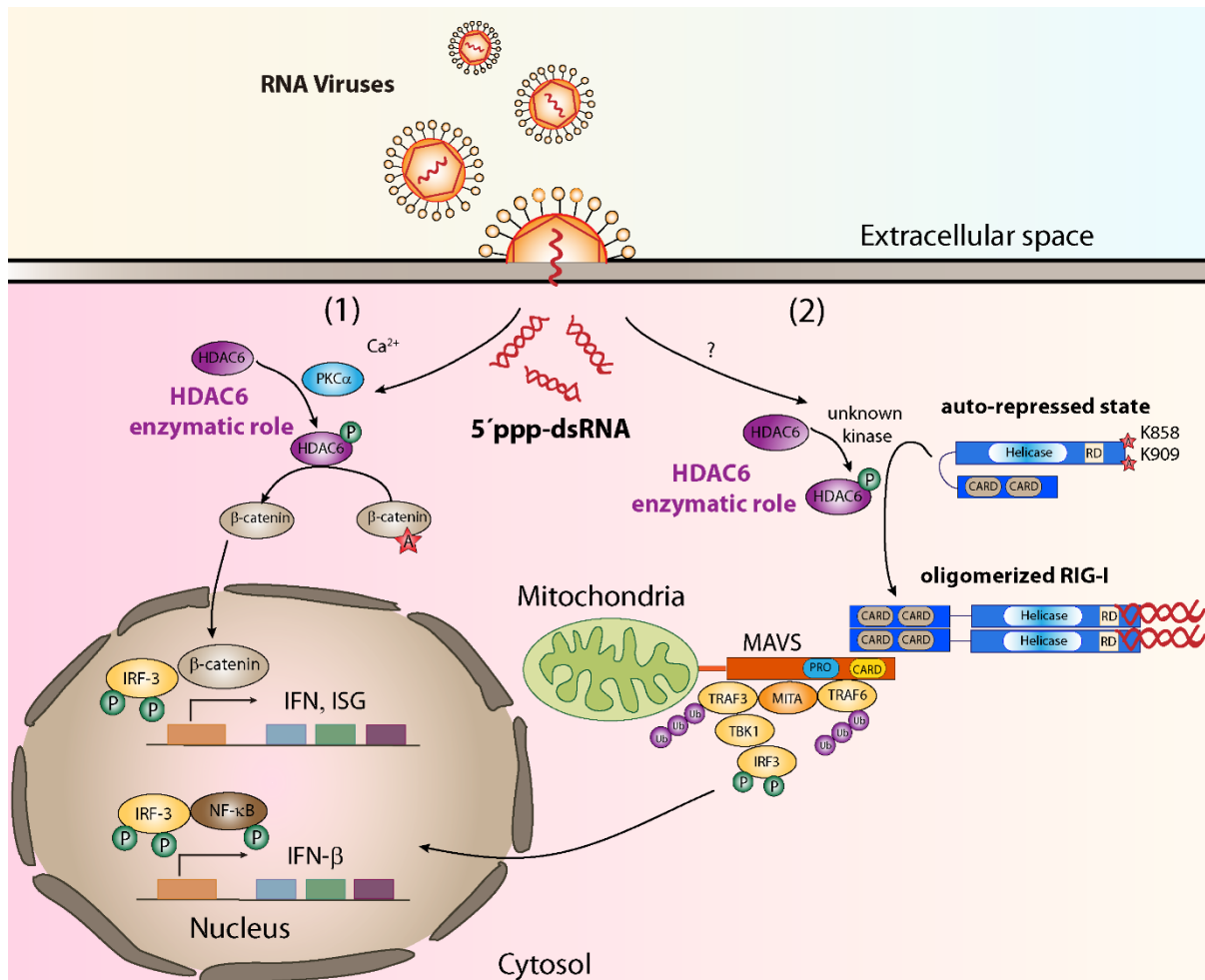
### 1.5.2. Role of HDAC6 during viral infections

Through acetylation, HDAC6 controls microtubule stability and consequently the kinesin and dynein-dependent transport of various cargoes. Cargo movement mechanisms are co-opted by different types of pathogens, including viruses and intracellular bacteria. Thus, HDAC6 becomes a crucial intermediary involved in viral fusion, entry, nuclear shuttling, replication, assembly and egress [249,165,93]. Likewise, HDAC6 controls the mechanisms involved in the elimination of these pathogens. For example, HDAC6 is involved in CD4<sup>+</sup> T lymphocytes function during the human immunodeficiency virus (HIV) infection [243]. The binding of the viral envelope glycoprotein gp120 to permissive CD4<sup>+</sup> T cells increases acetylated tubulin. HDAC6 overexpression inhibits tubulin acetylation, preventing viral envelop-mediated cellular fusion and infection, without affecting the expression and co-distribution of viral receptors. Consequently, the diminished enzymatic activity of HDAC6 favours HIV-1 infection and syncytia formation [243]. However, the activity of this protein in the case of influenza A virus (IAV) seems to play a dual role. On one hand, it has been described that viral capsids mimic unfolded-protein aggregates to take advantage of the host cell aggresome formation to disassemble and successfully eliminate viral capsids to continue the infection [15]. On the other hand, it has been observed that microtubules deacetylated by HDAC6 hamper the viral cycle, preventing trafficking of viral components through the cytoskeleton, which is necessary for viral assemble sites in the host plasma membrane and their posterior spreading to neighbouring cells [93]. Besides, caspase-3 cleaves HDAC6 in IAV-infected cells, removing its BUZ domain, although the consequence in IAV infection remains unknown [94].

Other HDAC6 substrates different from cytoskeletal proteins participate in viral pathogenesis like the HIV-1 transactivator of transcription protein (Tat) and the Retinoic acid Inducible Gene-I (RIG-I) [49,137,92,226]. HDAC6 regulates HIV viral replication by the deacetylation of Tat at transcriptional level, impairing the binding of Tat to cyclin T1, which inhibits viral transactivation [92,226]. In addition, HDAC6/apolipoprotein B m-RNA-editing enzyme-catalytic, polypeptide-like 3G'' (A3G) complex interacts with Viral infectivity factor (Vif) for its autophagic degradation, inhibiting HIV-1 spreading



[245]. Recognition of 5'ppp-dsRNA by RIG-I induces its self-oligomerization via repressor domain (RD) of two RIG-I molecules, initiating a conformational change. RIG-I specific deacetylation at residues K<sup>858</sup> and K<sup>909</sup> by HDAC6 induces RIG-I “signalling on” conformation, an essential step to engage Mitochondrial anti-viral-signalling protein (MAVS) and to activate the anti-viral response through Interferon responding factor 3 (IRF-3)-Interferon- $\beta$  (IFN- $\beta$ ) axis [137,49] (Figure 1.5). Protein kinase C- $\alpha$  (PKC- $\alpha$ ) is activated by calcium changes, phosphorylating HDAC6 during SeV infection [271]. This PTM enhances HDAC6 enzymatic activity and thereby, mediating deacetylation of  $\beta$ -catenin, which works as co-activator of IRF-3 transcription [271] (Figure 1.5)



**Figure 1.5. Proposed model of HDAC6 functions in pathogen sensing.** (1) Calcium changes triggered by viral entry activates PKC $\alpha$ , which phosphorylates HDAC6, increasing its deacetylase activity on  $\beta$ -catenin. This protein is imported in the nucleus together with IRF-3 to activate the interferon response. (2) After RNA viral infection, 5'ppp-dsRNA alerts to an unknown kinase, which activates HDAC6 by phosphorylation. HDAC6 deacetylates K<sup>858</sup> and K<sup>909</sup> residues of RIG-I, promoting its dimerization and binding of 5'ppp-dsRNA, which is necessary for the activation of MAVS signalling pathway. Then, IRF-3 and Nuclear factor- $\kappa$ B (NF- $\kappa$ B) initiate the interferon- $\beta$  genetic transcription to alert the anti-viral host response. Adapted from Zheng *et al* 2017 [270].

Protein kinase C- $\beta$  (PKC- $\beta$ ) is also able to phosphorylate HDAC6 and enhances its enzymatic function over  $\beta$ -catenin, promoting the transcriptional activation of IRF-3 and the subsequent production of type-I IFN in response to Toll-like receptors 3 and 4 (TLR-3 and 4) agonists [46]. HDAC6, through its BUZ

domain, directly associates with ubiquitinated NOD-like receptor (NLR) family, pyrin domain-containing 3 (NLRP3), down-regulating the activation of NLRP3 inflammasome independently of deacetylase activity [95]. Finally, the observation that HDAC6-deficient mice are more susceptible to RNA virus infections is in accordance with the observed increased resistance of overexpressing-*Hdac6*<sup>tg</sup> mice to avian H5N1 virus, highlighting the role of HDAC6 as a new regulator of the innate immune defence [249,93,165].

### 1.5.3. Role of HDAC6 during bacterial infections and TLR-activation

Recent evidence supports a role of HDAC6 in bacterial infections. Hence, bladder-infiltrating neutrophils of HDAC6-deficient mice during uropathogenic *Escherichia coli* (UPEC) infection display higher bacteria titres [132]. Recently, new molecular mechanisms have been characterized to be involved in inflammatory processes caused by bacterial TLR-agonists such as LPS, suggesting a wider implication of HDAC6 in innate immunity [171,261,46]. HDAC6-deficient mice are protected from LPS-induced septic shock compared to wild-type mice, due to a defective pro-inflammatory response in the absence of HDAC6 [46]. Inhibition of HDAC6 expression and HDAC6-deficiency cells reduce pro-inflammatory cytokines after TLR-stimulation, although it is not clear whether deacetylase activity is directly involved in all cases [171,261,46,137,49]. However, it has been observed that HDAC6 hampers the formation of MyD88-TRAF6 signalling complex, suppressing TLR-4 mediated activation [97]. Tat protein in primary mouse astrocytes induces a severe neuroinflammation, and the silencing of HDAC6 reduces levels of chemokines, cytokines and adhesion molecules, diminishing the activation of NF- $\kappa$ B and AP-1 Transcription Factors (TF) responsible of pro-inflammatory response [226]. Therefore, increased expression of HDAC6 protein after TLRs activation and viral infections could serve as a protective cellular strategy to deacetylate microtubules and actin networks for avoiding microbial spreading [249,226].

## 2. *Listeria monocytogenes*

*Lm* was identified in Cambridge in 1926 as a lethal disease which displayed a pronounced monocytosis, an increased number of monocytes in circulating blood, in rabbits and guinea pigs caused by a Gram-positive bacterium referred to as *Bacterium monocytogenes* [163]. Another report in South Africa described also a Gram-positive bacterium responsible of Tiger River Disease in wild gerbils. Prominent necrotic lesions in liver were described and the microorganism was called *Listerella hepatolytica*, in honour to the surgeon Joseph Lister. New studies concluded both pathogens were the same, and the final name was designated as *Listeria monocytogenes*.

The genus *Listeria* contains 17 species which are able to grow at low temperatures and are very resistant to environmental stresses, such as low pH and high salt concentrations, remarking the



concerns for the food industry [181]. Besides, *Listeria* spp. thrives in a huge range of environments, can survive in water, soil and detritus and is able to infect mammalian hosts due to a particular adaptability to cross several host barriers, mediated by a vast number of regulatory factors. *Lm* is a non-spore-forming facultative anaerobic rod-shaped bacterium, which can be flagellated under 30°C. The systematic sequencing of the bacterial genome of *Lm* has revealed a genome size of 2,944,528 base pairs coding for 2,853 proteins [161,73]. However, the most important virulence factors of this pathogen has been characterized using biochemical and molecular genetic tools. Besides, murine listeriosis has been widely used as a forefront model for the study of host-pathogen interaction and innate and adaptive immune responses to intracellular bacterial infection over the past 20 years [185,80,175,242].

## 2.1. *Listeria monocytogenes* pathogenesis

Although *Lm* was first reported to infect rabbits and gerbils, this bacterium has an unusually broad host range, infecting almost every mammals, including humans [33]. This intracellular *Gram*-positive bacterium causes listeriosis, a self-limiting gastroenteritis in healthy individuals, but can cause severe and systemic infection in immunocompromised individuals, the elderly, pregnant women and newborns due to its ability to cross the blood-brain barrier and placenta [185,214]. Immunocompromised individuals suffer septicaemia and meningitis, pregnant women in third trimester develop chorioamnionitis and infectious of the foetus which can lead to septic abortion [12]. Humans are exposed to the infection of this bacterium by ingesting contaminated food, usually unpasteurized or incompletely cooked meats [214]. Although *Lm* is considered a food-borne intracellular bacteria that causes a moderately low number of infections per year (listeriosis to 23.150 people worldwide in 2010), the mortality among infected individuals is strikingly high (30%) being responsible of approximately 6,900 deaths every year [50].

## 2.2. Infective cycle of *Listeria monocytogenes*

### 2.2.1. Bacterial entry

Despite most studies have used intravenous or intraperitoneal inoculation of *L. monocytogenes*, the natural route of infection is through the gastrointestinal tract [125]. *Lm* can replicate in the cytosol of several host cell types such as epithelial cells and macrophages [194]. The efficiency of bacteria uptake can vary depending of cellular type, e.g. macrophages and other phagocytic cells internalize up to 20 bacteria per cell, whereas fibroblast cell lines internalize less than one bacterium [33]. *Lm* co-opts the cellular receptor-mediated endocytosis machinery to entry into non-phagocytic cells, while *Lm* entry in phagocytic cells is actively mediated by immunoglobulin receptor-mediated engulfment of opsonized bacteria [43]. After the ingestion of this pathogen, intestinal epithelial cells are infected by

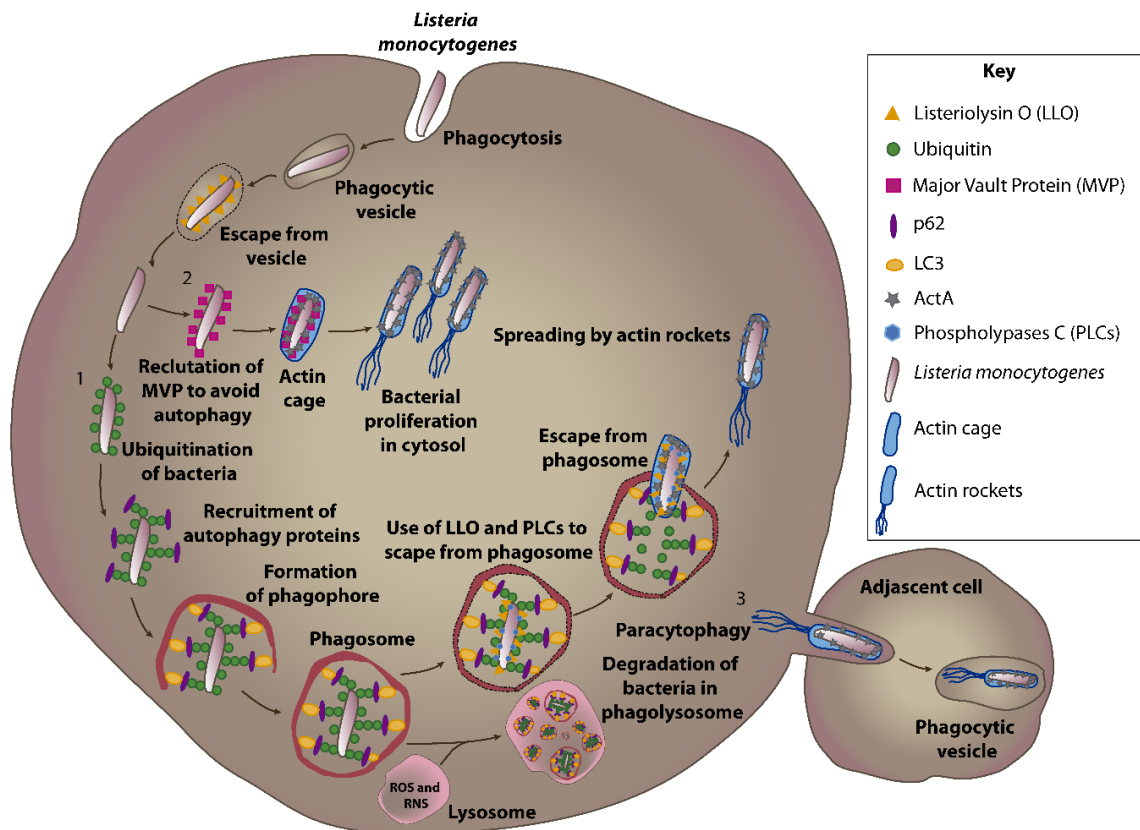
the interaction of the surface bacterial protein internalin A (InIA) with the extracellular domain of the epithelial E-cadherin of the host cell through a zipper-like mechanism [69,188]. The ability of this bacterium to infect intestinal epithelial cells is quite variable among mammals [111]. For example, mice have a limited susceptibility to intestinal infection due to a single amino-acid difference between human and mouse E-cadherin [124]. Upon this interaction, *Lm* is able to traverse the epithelial cell layer and disseminate in the bloodstream towards other organs. This bacterium has tropism in particular for spleen and liver, where not only is internalized by splenic and hepatic macrophages, but also by hepatocytes using the bacterial protein internalin B (InIB) to interact with the hepatocyte growth factor receptor (HGFR), mediating the internalization via Phosphatidyl Inositol 3-phosphate (PI3)-kinase activation [42,221]. Hence, internalins A and B are considered strategic tools of *Lm* for adhesion and invasion of host cells, increasing the level of bacteria internalization by non-professional phagocytic cells. The family of internalins is composed of 25 members, can be anchored to the cell wall, associated or secreted for regulating a vast number of virulence-related function [22]. InIC seems to affect the rigidity of the cytoskeleton and innate immune signalling, InIP is implicated in placental invasion and InIJ has unknown function and is only expressed *in vivo* [201,211,68]. In addition to InIA and B, both fibroblasts and monocytes use the high affinity immunoglobulin gamma Fc receptor I (FCGR1A or CD64) as *Lm* entry receptor without the opsonisation step [189].

### 2.2.2. Bacterial tools to evade the autophagy system

From the first hours of infection, professional phagocytic cells trap bacteria in the blood and target organs, spleen and liver, exerting a degree of control on bacterial growth [236]. Liver macrophages and hepatocytes are the preferential hosts for *Lm*, although other cell types are also infected such as epithelial cells and other phagocytic cells. Upon entry, bacterium is internalized inside a phagocytic vacuole and *Lm* is eliminated by fusion of the phagosome with lysosomes. Interestingly, phagosome-contained bacteria are also eliminated by the action of reactive oxygen species (ROS) and nitric oxide (NO), respectively produced by NADPH oxidase 2 (NOX2) and inducible NO synthase (iNOS) [144][222](Figure 1.6). However, the majority of bacteria escape from the phagosome into the cytoplasm through the action of the cholesterol dependent family of cytolysins listeriolysin O (LLO) and two other phospholipases C (PLCs), a phosphatidylinositol-specific PLC (PlcA) and a broad spectrum one (PlcB), which causes vacuolar rupture [17,71,148,35,23]. In HeLa cell line, escape from the vacuole is exclusively mediated by both phospholipases [148].

*Lm* is able to replicate in spacious *Lm*-containing phagosomes (SLAPs) in some macrophages [23]. The formation of SLAPs depends on microtubule associated protein light-chain 3 (LC3)-mediated phagocytosis combined with intermediate levels of LLO expression, which dampen the phagosome acidification, resulting in *Lm* proliferation without phagosomal rupture [120]. ActA is also an essential

bacterial protein to evade the cell host autophagy. In this regard,  $\Delta actA$  bacteria is ubiquitinated and subsequently recognized by ubiquitin-binding receptors p62 and nuclear dot protein 52 (NDP52) and incorporated in LC3<sup>+</sup>-compartments [266,160]. Indeed, cellular factors that alter ActA localization on the bacterial surface could dampen cell-to-cell spreading of *Lm*, such as the case of type I IFN receptor 1 (IFNAR1)-deficient mice, which display lower bacterial burden [63,8,230,182]. Cell host Major Vault protein (MVP) is hijacked by bacterial InlK to avoid ubiquitination and the subsequent autophagy [60].

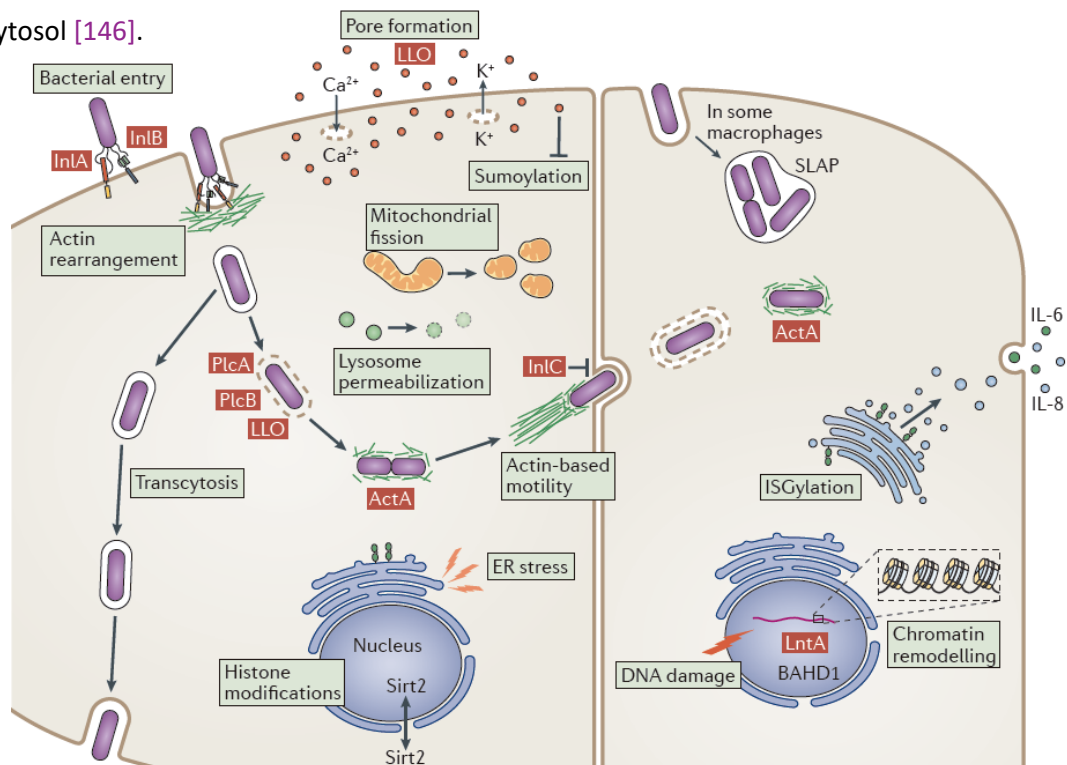


**Figure 1.6. Overview of infective cycle of *Lm* in a phagocytic cell.** *Lm* enters the cell by membrane vesicle phagocytosis. Using LLO is able to escape from the vesicle to the cytoplasm. **1.** Once in the cytoplasm, bacteria are rapidly ubiquitinated and consequently recognized by autophagy proteins, such as LC3, p62, ATG family. These proteins are required to initiate the phagophore formation, a double-membrane vesicle where ubiquitinated bacteria are engulfed. Inside phagosome, the action of ROS, Reactive Nitrogen Species (RNS) and lytic enzymes of the lysosome contribute to bacterial degradation in a continuous process of phagosome-lysosome pH acidification. However, a percentage of bacteria can avoid this process by the expression of LLO and two phospholipases C (PlcA and B), which are activated with the lower pH, allowing *Lm* to form a pore in the phagosome membrane to evade its degradation. By the use of ActA, *Lm* travels through the cytosol polymerizing cellular actin, drawing characteristic acting comets. **2.** Besides, this bacterium can avoid the autophagy by another mechanism, expressing InlK, which recruit the mammalian MVP to block bacteria ubiquitination. The use of ActA protein is also responsible of the formation of actin cage, to avoid autophagy. Once in the cytosol, *Lm* proliferates due to permissive environmental conditions. **3.** Moreover, actin-polymerizing bacteria are able to infect neighbouring cells without leaving the cytoplasm, making long actin rockets in a process named paracytophagy.

### 2.2.3. *Lm*-induced cellular changes

In the cytoplasm, *Lm* replicates in a more benign and nutrient-rich environment and is able to infect neighbouring cells [193,28,178]. *Lm* is able to replicate and survive in cell host cytosol without causing

cell death because of its unique intracellular lifestyle. This hallmark is due to additional functions of LLO, different from its pore-forming activity, which cause multiple changes in organelle morphology and function before LLO plasma membrane entry and after its cellular internalization [83] (Figure 1.7). LLO causes a non-cannonical mitochondrial fission, independent of dynamin-related protein 1 (DRP1) and accompanied by smaller and rounded mitochondria, which seems to enhance bacterial division [227-229]. Upon infection, this cytolysin induces Endoplasmic Reticulum (ER) stress and Unfolded Protein Response (UPR), responsible for upregulating chaperones, blocking translation into the ER and increasing ER folding capacity [192]. Animals with a defective UPR succumb to *Lm* infection [106]. Finally, LLO alters lysosomal membrane permeability and homeostasis, causing a release of cathepsins into cytosol [146].



**Figure 1.7. *Lm* infective cycle in a non-phagocytic cell and in some macrophages.** *Lm* enters epithelial cells through receptor-mediated endocytosis and the majority of bacteria escape from the vacuole. By transcytosis, vacuole-contained *Lm* can pass across the cell in globet cells, whereas *Lm* can replicate in spacious *Lm*-containing phagosomes (SLAPs) in some macrophages. Potent virulent factors as LLO, PlcA and PlcB are involved in vacuolar escape. Upon vacuolar escape, *Lm* is able to polymerize actin by using the actin-assembly-inducing protein ActA, enhancing the spreading to neighbour cells. The pore-forming activity of LLO triggers numerous changes in host cell, such as histone modifications, desumoylation, mitochondrial fission, ER stress and lysosomal permeabilization. Host gene expression is controlled by LntA of *Lm*, which interacts with BAHD1 complex to derepress ISGs. This provokes Sirt2 shuttle into the nucleus to deacetylate histone 3 at K<sup>18</sup>, that changes chromatin packing and the subsequent gene expression and DNA damage. The host cell fights against infection by upregulating antibacterial effectors, such as ISG15 and its covalent incorporation to ER and Golgi proteins (a process namely ISGylation), which modulates cytokine secretion. From Radoshevich *et al* 2018 [198].

However, the host cell fights against infection by upregulating IFNs, cytokines and antibacterial effectors, such as IFN-stimulated genes (ISGs) [189]. In this regard, an ubiquitin-like protein, called IFN-stimulated gene 15 (ISG15), is induced during *Lm* infection and its covalent incorporation to newly-

synthesized proteins of ER and Golgi (namely ISGylation), increases cytokine secretion to counteract infection [200]. *Lm* dampens sumoylation of host cell proteins by degradation of SUMO-conjugating enzyme UBC9 required for this kind of PTM [204,96]. *Lm* is able to mimic receptor-mediated endocytosis engagement, promoting the phosphorylation and ubiquitination of the cytosolic domain of receptors E-cadherin and Met and their subsequent internalization, co-opting the clathrin machinery of the host cell [248,26]. *Lm* also promotes changes in histone PTMs, which changes the chromatin packaging and the subsequent access of transcription factors [81,82]. For instance, cell host sirtuin-2 is shuttled to the nucleus to deacetylate histone 3 at K<sup>18</sup> [67]. A nucleomodulin-type of bacterial virulence factor namely nuclear-targeted protein A (LntA), controls host gene expression through its interaction with the bromo adjacent homology domain-containing 1 protein (BAHD1) complex to derepress ISGs [122,123].

#### **3.2.4. Intracellular and extracellular motility**

Wiskott Aldrich Syndrome protein (WASP) is a nucleation promoting factor that interacts with the actin-related protein 2/3 (ARP2/3) complex to induce actin polymerization on host cell. *Lm* contains a surface-anchored protein, called actin-assembly inducing protein (ActA), which mimicks WASP to enable bacterial propulsion to neighbouring cells through the directional assembly of actin filaments (actin rockets) [113,121,37,28,225]. The canonical ARP2/3 complex is formed by 7 subunits: ARP2 and 3 and ARPC1-5, and *Lm* co-opt different combinations of these subunits depending on the type of actin polymerization or branching required in every step, such as bacterial entry or actin comet tail formation [117]. However, vacuole-contained *Lm* can pass across the cell cytoplasm in globet cells by transcytosis, acting as a vacuolar bacterium for avoiding cytosolic immune detection [174]. *Lm* can also spread from cell to cell elongating filopods to adjacent cells without exiting the intracellular compartment by a process called paracytophagy, which exploits host cell efferocytosis for evading immune detection [157]. LLO induces membrane damage in actin protrusions, exposing phosphatidylserine, which is usually found in the inner membrane of the host cells, therefore adjacent macrophages capture protrusion-containing *Lm* [44].

#### **3.2.5. Cellular immune response to infection**

During food-borne infection, the majority of *Lm* is extracellularly attached to the surface of activated monocytes, although these cells are not a proliferation niche for bacteria [100]. However, monocytes increase CD64 expression during their transition to macrophages, which *Lm* uses as an entry receptor, thereby allowing intracellular bacteria to spread to the liver and spleen [101,41]. Resident Kupffer cells phagocytose *Lm* in the liver and the posterior necroptosis of these macrophages generates specific inflammatory mediators that activate liver repair processes, the mobilization of bone-marrow-derived

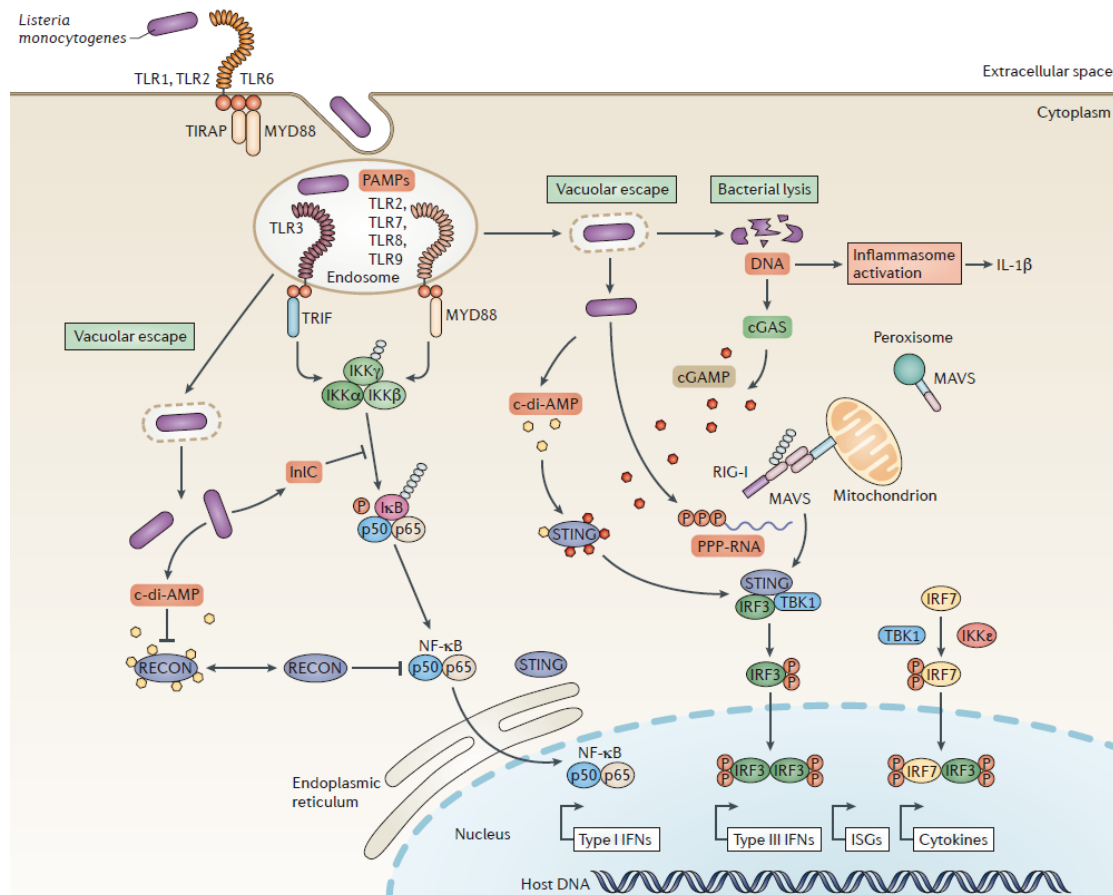
monocytes and awake the immune system [25]. Nevertheless, the early control of *Lm* burden largely depends on the innate immune response occurring in the spleen, which relies on two main cell populations of dendritic cells (DCs). On one hand, a subset of monocyte-derived DCs namely TNF/iNOS-producing DCs (Tip-DCs) has the ability to produce TNF- $\alpha$  and NO [219,236]. On other hand, CD8 $\alpha^+$ splenic conventional DCs (cDCs) are responsible for the final resolution of *Lm* infection through the presentation of bacterial-derived antigens to specific CD8 $^+$ T cells to induce cytotoxicity [86,65,102]. Moreover, it has been reported that CD8 $\alpha^+$  cDCs are the first splenic population responsible for the phagocytosis of bacteria *in vivo* [170]. However, the host cell of cytosolic *Lm* is able to develop a specific CD8 $^+$ T cell response, which is crucial for the control of infection [40,154,79,10]. It has been reported that mutant LLO *Lm* strains are unable to develop CD8 $^+$  T cell adaptive response and, accordingly, CD8 $^+$  T cell memory against this pathogen [154,79,10].

### 3.2.6. Innate immune sensing of *Lm*

The response of dendritic cells (DC) to live *Lm* is mediated by Toll-like receptors (TLRs), nucleotide-binding oligomerization domain (NODs)-like receptors (NLRs), and other cytosolic receptors and involves two signalling pathways: TLR-dependent and independent signalling [198,199]. *Lm* pathogen-associated molecular patterns (PAMPs) are sensed on the cellular surface by TLRs 1, 2 and 6, whereas phagosomal-contained bacteria activate endosomal TLRs 2, 3, 7, 8 and 9. TLR-3 mediates TRIF-dependent signalling pathways and the rest of TLRs mediates a MyD88-dependent response. Both cellular pathways activate NF- $\kappa$ B signalling for the initiation of anti-bacterial host response. After phagosomal escape, *Lm* activates the inflammasome and cyclic GMP-AMP synthase (cGAS), which produces a host-specific cyclic dinucleotide (CDN) called cGAMP, that also activates the sensor stimulator of interferon (IFN) genes (STING) [237]. Passive release or active secretion by *Lm* of the bacterial 5'triphosphate RNA (5'-pppRNA) is sensed by retinoic acid-inducible gene I protein (RIG-I), which triggers the mitochondrial and peroxisomal forms of anti-viral-signalling protein (MAVs) [1,77]. These synergic pathways upregulate IFNs, cytokines and anti-bacterial effectors such as IFN-stimulated genes (ISGs). At early stage of infection, bacterial DNA is sensed by the cytosolic surveillance pathway, that includes STING, the serine/threonine-protein kinase TBK1 and the IFN regulatory factors (IRFs) 3 and 7 [4,199]. This pathway induces the expression of ubiquitin-like protein ISG15, independently of IFN, and the conjugation of ISG15 to newly synthesized Endoplasmic Reticulum (ER) and Golgi proteins as a cellular mechanism for tagging proteins after infection [200]. Besides, *Lm* secretes a small dinucleotide namely cyclic di-AMP (c-di-AMP) that directly triggers STING pathway, leading to IFN $\beta$  production and activation of downstream signals that control the transcription of IFN target genes essential for antiviral and antibacterial responses [255,89,4,257]. However, the activation of innate immune pathways by c-di-AMP negatively activates T cell-mediated responses, reducing the clearance



of *Lm* after a reinfection [4]. Additionally, the liver enzyme called aldo-keto reductase family 1 member C13 (AKR1C13 or RECON) binds to c-di-AMP with higher affinity than STING. STING affinity for host CDNs is stronger than for c-di-AMP, balancing the signalling pathways triggered by c-di-AMP on favour of RECON [152]. Although TLR-1 and 2 and NOD1 are activated by *Lm* PAMPs to stimulate NF- $\kappa$ B signalling, RECON negatively regulates this pathway [152]. However, after bacterial CDN-RICON binding, the enzyme is inactivated, recovering the NF- $\kappa$ B signalling and Nitric Oxide (NO) production [152]. The complete molecular innate immune response to *Lm* is summarized in **Figure 1.8**.



**Figure 1.8. Innate immune sensing of *Lm*.** *Lm* is sensed by TLRs 1, 2 and 6 in the plasma membrane and by TLRs 2,3,7,8, and 9 in endosomes, triggering the TLR-signalling pathway and the nuclear import of NF- $\kappa$ B to induce genetic transcription of IFNs, cytokines and ISGs. Upon vacuolar escape of *Lm*, the cytosolic surveillance pathway is activated by bacterial DNA, 5'pppRNA and c-di-AMP, inducing STING, TBK1, RIG-I, mitochondrial and peroxisomal MAVs, IRFs 3 and 7 and RECON. From Radoshevich *et al* 2018 [198].

Although, the function of HDAC6 has been described in autoimmune, inflammatory and viral disease models, there is not much information about its role in the regulation of innate immunity during the infectious processes by non-viral pathogens, such as bacteria and protozoan. For this reason, this doctoral thesis is focused in the study of the role of HDAC6 in the innate immune host defence against *Listeria monocytogenes* infection and tentative molecular mechanisms involved.





# Objectives



## 2. OBJECTIVES

The general objective of this thesis aims to investigate the functions of HDAC6 during the innate immune response to the intracellular bacteria *Listeria monocytogenes* (*Lm*). The specific objectives are the following:

### 1. Analysis of HDAC6 contribution to cellular mechanisms that eliminate intracellular bacteria by phagocytic cells

- 1.1. Effect of HDAC6 absence in the intracellular pathogen clearance of dendritic cells
- 1.2. Measurement of *Lm* load in HDAC6-deficient myeloid cells
- 1.3. Determination of *in vivo* bacterial burden
- 1.4. Evaluation of cellular clearance mechanisms of *Lm*
- 1.5. Consequence of the lack of HDAC6 in the autophagy of *Lm*
- 1.6. Effect of HDAC6 absence in the accumulation of autophagy marker p62
- 1.7. Mechanism by which HDAC6 regulates autophagy of *Lm*

### 2. Study of the role of HDAC6 in the activation of dendritic cell functions by bacterial infection and stimulation of TLRs

- 2.1. Determination of pro-inflammatory gene expression
- 2.2. Effect of HDAC6 in pro-inflammatory cytokine secretion after *Lm* infection
- 2.3. Influence of HDAC6 in the microbicidal activity of iNOS
- 2.4. Study of the expression of MHC co-stimulatory molecules on the membrane
- 2.5. Function of HDAC6 in the activation of MAPK- and mTOR-signalling pathways
- 2.6. Pro-inflammatory cytokine secretion after stimulation with TLR agonists
- 2.7. Consequence of HDAC6 defect in the activation of TLR-signalling pathway
- 2.8. Evaluation of the HDAC6 role in the *in vivo* TLR-dependent inflammatory response



# Material and Methods



### 3. MATERIAL AND METHODS

#### 1. Ethical Statement

##### 1.1. Mice procedures

Mice were housed under specific pathogen-free conditions at the Centro Nacional de Investigaciones Cardiovasculares Carlos III (CNIC), and experiments were approved by the CNIC Ethical Committee for Animal Welfare and by the Spanish Ministry of Agriculture, Food, and the Environment. Animal care and animal procedures license were reviewed and approved by the local Ethics Committee for Basic research at the CNIC Ethical Committee for Animal Welfare and the Órgano Encargado del Bienestar Animal (OEBA) del Gabinete Veterinario de la Universidad Autónoma de Madrid (UAM). This committee approved the document with an associated identification number PROEX 158/15 (CNIC 04/15).

##### 1.2. Human samples

Buffy coats of healthy donors were received from the Blood Transfusion Center of Comunidad de Madrid, and all donors signed their consent for the use of samples for research purposes. All the procedures using primary human cells were approved by the Ethics Committee of the Hospital Universitario de la Princesa (HUP).

#### 2. Mice

*Hdac6*<sup>-/-</sup> mice were generated through targeting of exons from 10 to 13 by inserting a neomycin (Neo) and zeocin (Zeo) cassette, resulting in the disruption of the first catalytic domain of HDAC6 [70]. These mice were intercrossed on a C57BL/6 background to generate sex and age matched wild-type (wt) and knockout (KO). Mice used in this work were generously donated by Dr. Tso-Pang Yao (Department of Pharmacology and Cancer Biology, Duke University, Durham, North Carolina, USA) in a mixed background Sv129-C57BL/6. After that, mice were inter-crossed on a C57BL/6 background 8 times more to ensure a purer C57BL/6 background. F8 inter-crossed on C57BL/6 background ensures over 90% of the latter background.

#### 3. Genotyping of mice

In order to genotype these mice and check the homozygosity of the deletion of the first catalytic domain of HDAC6, a PCR was performed with the two pairs of primers of the **Table 3.1**.

**Table 3.1. Genotyping primers.** Table of primers used to genotype mice disclosed by gene name, sequence 5'-3' and size of the amplification product. (bp: base pair).

Gene	Sequence (5'-3')	Amplification product
<b>Int-9</b>	CTGGTTCGTCTGAAG	350 bp
<b>Exo-10</b>	GTGGACCAGTTAGAAGCC	
<b>Zeo-1</b>	CCATGACCGAGATCGGCGAGC	250 bp
<b>Zeo-3</b>	CGTGAATTCCGATCATATTCAAT	

The pair of primers Int-9/Exo-10 amplifies the *wt* allele (350 bp), whereas the pair of primers Zeo-1/Zeo-3 amplifies the knockout allele (250 bp). These two pairs of primers were employed in PCR reactions using the genomic DNA from mouse tails as template DNA. Genomic DNA was obtained from mouse tails using the protocol of the reactive REExtractc-N-Amp tissue PCR kit (Sigma). As controls, genomic DNA from *wt*, heterozygous and homozygous mice were used. The amplification protocol is showed in the **Table 3.2**.

**Table 3.2. PCR amplification protocol.** Table of PCR cycles for genotyping mice disclosed by phase, temperature, time and number of cycles.

Phase	Temperature (°C)	Time (min, s)	Cycles
<b>1</b>	95	3 min	1x
	95	30 s	
<b>2</b>	55	30 s	30x
	72	1 min	
<b>3</b>	72	10 min	1x

## 4. Bacteria strains

### 4.1. *Listeria monocytogenes* strains

We used the *Listeria monocytogenes* EGD (BUG 600) strain, provided by Dr. Esteban Veiga (Centro Nacional de Biotecnología, CNB, Madrid). Red Fluorecent Protein-expressing *Listeria monocytogenes* (RFP-*Lm*) was provided by Dr Carlos Ardavín's laboratory (Centro Nacional de Biotecnología, CNB, Madrid). BUG600 strain was grown in Brain Herat Infusion (BHI) broth.

### 4.2. *Salmonella enterica* strain

*Salmonella enterica* serovar Thyphimurium strain SL1344 was provided by Dr. J. Garaude (Centro Nacional de Investigaciones Cardiovasculares, CNIC, Madrid). SL1344 strain was grown in Luria-Bertani (LB) broth supplemented with 50 µg/ml streptomycin (Sigma).

### 4.3. *Staphylococcus aureus* strain

*Staphylococcus aureus* 132 (*S. aureus*) was purchased from Invitrogen. *S. aureus* strain was grown in BHI broth.



#### 4.4. *Escherichia coli* K12 strain

*Escherichia coli* K12 strain DH5 $\alpha$  (*E. coli*) was purchased from Invitrogen. DH5 $\alpha$  strain was grown in LB broth supplemented with 50  $\mu$ g/ml streptomycin (Sigma).

For phagocytosis experiments, *Lm* and *S. aureus* were grown overnight in BHI broth and *E. coli* and *S. Typhimurium* in LB broth with shaking, diluted 1/50, and grown until log-phase (optical density 0.8-1.2 at 600 nm) without shaking. Bacteria were washed with phosphate-buffered saline (PBS) to remove LB and BHI salts before addition to cells.

### 5. Cell culture

#### 5.1. HEK293T cell line

The HEK293T cell line came from human embryonic kidney transformed with the large T antigen of SV40. The HEK293T cell line (ATCC) was cultured in Dulbecco's Modified Eagle medium (MEM) (Sigma) containing 10% FBS (Invitrogen), 2 mM L-glutamine, 100 mg/ml penicillin and 100 mg/ml streptomycin.

#### 5.2. HEK Blue hTLR2 cell line

HEK Blue hTLR2 cell line (Invivogen), the HEK293 cell line expressing human TLR2, CD14 and NF- $\kappa$ B-SEAP (secreted embryonic alkaline phosphatase) reporter gene was cultured in DMEM medium (Sigma) containing 10% FBS (Invitrogen), 2 mM L-glutamine, 100  $\mu$ g/ml Normocin (Invivogen) and 1X HEK-Blue Selection (Invitrogen).

### 6. Generation of Bone Marrow-Derived Dendritic Cells (GM-CSF) and Macrophages (M-CSF)

Mouse primary bone marrow-derived dendritic cells (BMDCs) and macrophages (BMDMs) were obtained from bone marrow cell suspensions after culture on non-treated 150-mm Petri dishes in complete Roswell Park memorial institute-1640 medium (RPMI 1640) supplemented with 10% FBS, 2 mM L-glutamine, 100 mg/ml penicillin, 100 mg/ml streptomycin, 50 mM 2-ME, and 20 ng/ml granulocyte-macrophage colony-stimulating factor (GM-CSF, PeproTech, London, U.K.) for BMDCs and macrophage colony-stimulating factor (30% mycoplasma-free L929 cell supernatant, NCBI Biosample accession number SAMN00155972) for BMDMs. BMDCs were collected at day 9 and BMDCs were characterized as CD11c<sup>+</sup>MHC-II<sup>+</sup>Gr-1<sup>-</sup> cells by flow cytometry. BMDMs were collected at day 6 and BMDMs were characterized as CD11b<sup>+</sup>F4/80<sup>+</sup> or CD11b<sup>+</sup>CD64<sup>+</sup> cells.

## 7. Generation of Bone Marrow-Derived Dendritic Cells (FLT3L)

Bone marrow cell suspensions were culture on treated 6 well plates in complete RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, 100 mg/ml penicillin, 100 mg/ml streptomycin, 50 mM 2-ME, and 150 ng/ml Fms-related tyrosine kinase 3 ligand dendritic cells (FLT3L, PeproTech, London, U.K.). After 9-11 days of differentiation cells were collected to be characterized by flow cytometry as CD11c<sup>+</sup>B220<sup>-</sup>CD11b<sup>+</sup>CD24<sup>-</sup> (60% of the culture) and CD11c<sup>+</sup>B220<sup>-</sup>CD11b<sup>+</sup>CD24<sup>+</sup>(40%).

## 8. Obtainment of Thioglycollate-Elicited Macrophages (TEMs)

Mice received peritoneal injections with 1ml 4% thioglycollate (TG). The peritoneal exudate was collected after 4 days and cultured in complete RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, 100 mg/ml penicillin, 100 mg/ml streptomycin, and 50 mM 2-ME. To enrich the culture for macrophages, non-adherent cells were eliminated after a few hours by washing five times with warm PBS and gentle swirling. These cells expressed CD64 macrophage marker in more than 90% after this enrichment.

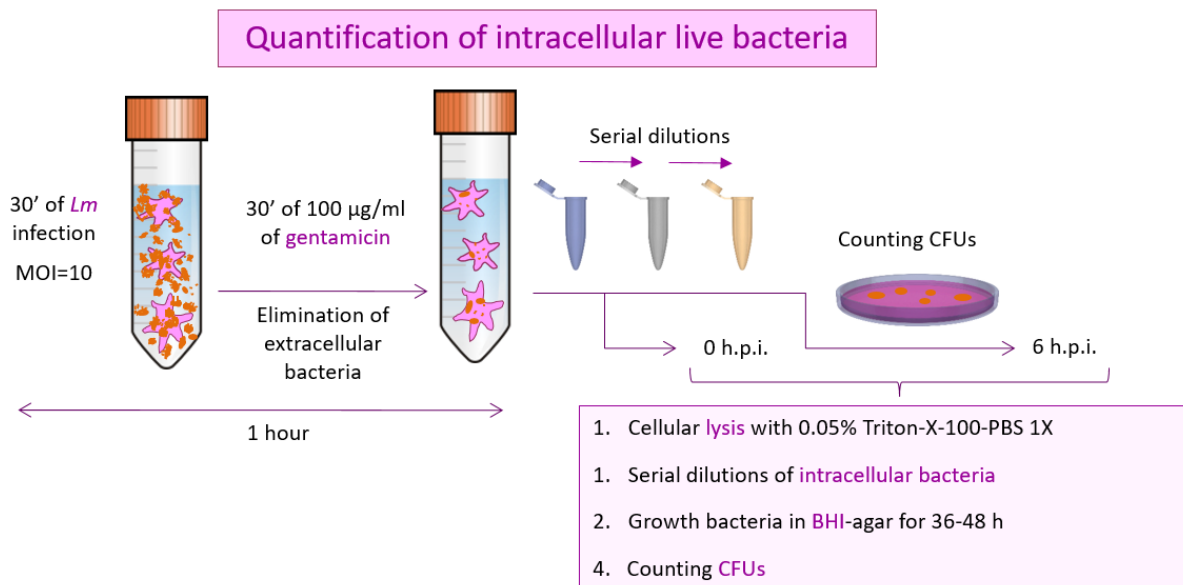
## 9. Obtainment of human monocyte-derived Dendritic Cells (moDCs)

Peripheral blood mononuclear cells (PBMCs) from Buffy coats of healthy donors were isolated using Biocoll separating solution (Millipore) by centrifugation at 700 g 30 min at RT. Monocytes were purified from peripheral blood mononuclear cells (PBMCs) by an adhesion step at 37°C in incomplete RPMI 1640 medium during 1 h. Non-adherent cells were removed and adherent monocytes were washed three times with warm 1x PBS to remove residual PBMCs. Monocytes were cultured in complete RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, 100 mg/ml penicillin, 100 mg/ml streptomycin, 500 U/ml IL-4 (R&D) and 500 U/ml GM-CSF (Immunotools) for 6 days. Fresh medium and cytokines were added every 48 h to differentiate monocytes to immature human dendritic cells. Cells were characterized by flow cytometry as HLA-DR<sup>+</sup>CD3<sup>-</sup>DC-SIGN<sup>+</sup>CD14<sup>+</sup>CD11c<sup>+</sup>. Activation of dendritic cells was induced with Pam2GSK4, Pam3GSK4 and HKLM for 30 min (Invivogen).

## 10. *In vitro* *Lm*-infection of BMDCs, BMDMs and TEMs

Cells were incubated with *Lm* and assessed for survival to gentamicin exposure [247]. Detailed, cells were infected with *Lm* at a multiplicity of infection (MOI) of 10 for 30 min at 37°C and 5% CO<sub>2</sub>. *Lm* is a facultative pathogen able to growth in culture medium. To prevent extracellular replication, the aminoglycoside antibiotic gentamicin was added to the medium after bacterial internalization. This antibiotic is bactericidal for extracellular bacteria at high concentrations and does not have any significant effect in intracellular ones during the first 8 hpi [247]. Hence, to determine the number of bacteria entering the cells, extracellular bacteria were killed by treatment with 100 µg/ml gentamicin

(Sigma-Aldrich, St. Louis, MO) for an additional 30 min at 37°C. Then, infected cells were washed with PBS three times and lysed with 0.05% Triton X-100 (Sigma-Aldrich, St. Louis, MO) in distilled water. Serial dilutions were seeded on brain-heart infusion (BHI) agar plates and colony-forming units (CFUs) were counted after 36-48 h. Quantification method of intracellular live bacteria is represented in the following scheme (Figure 3.1).

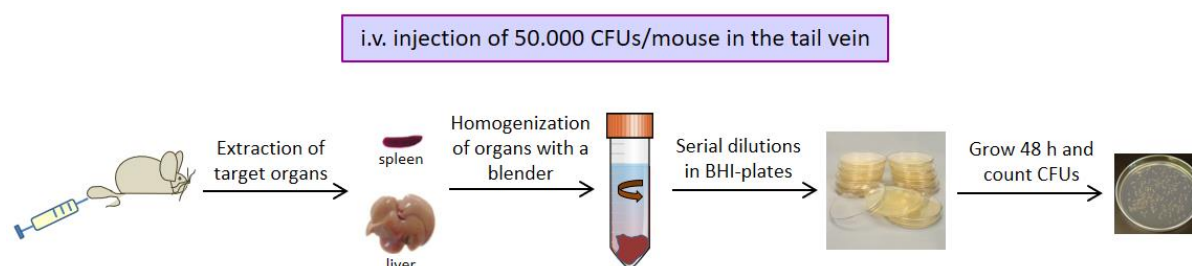


**Figure 3.1. Quantification of intracellular live bacteria in myeloid cells.** Myeloid cells are infected with *Lm* using a MOI of 10 during 30 min, followed by the addition of gentamicin during other 30 min. Cells are washed with PBS to eliminate the antibiotic and lysed with detergent at different experimental points (e.g. 0 and 6 hpi). Serial dilutions and not diluted sample are grown in BHI-agar plates for 36-38 h. At this time colony-forming units (CFUs) can be counted.

## 11. *In vivo* *Lm* systemic infections

*Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> mice were intravenously injected with *Listeria monocytogenes* EGD (125,000 CFUs/mouse resuspended in 200 µl of PBS 1x) using a 29-gauge needle. For survival experiments mice were monitored twice a day in order to detect casualties during 15 days of infection.

After 12, 24, 48 and 72 hpi, mice were perfused with 1X PBS to clean blood from organs and spleens and livers were weighted. To determine bacterial load, spleens and livers were digested with 0.1 mg/ml type IV collagenase and 0.5 mg/ml DNase I (Roche, Mannheim, Germany) for 30 min at 37°C. After digestion, organs were homogenized in 70 µm filters and red blood cells were lysed with ammonium chloride potassium lysis buffer (ACK, Sigma). Splenic cell suspensions were resuspended in PBS and cells were counted. Serial dilutions were grown on BHI agar plates. CFUs were counted after 36-48 h of incubation at 37°C. CFUs were calculated by cell number and by gram per organ. The model of quantification of intracellular bacteria in mouse target organs is illustrated in Figure 3.2.



**Figure 3.2. Quantification of intracellular live bacteria in mouse target organs.** Mice are infected with *Lm* by intravenous injection of 125.000 CFUs/mouse. Organs are extracted and homogenized using enzymatic digestion in combination with mechanical force on 70  $\mu$ m with the piston of a syringe. Cell suspensions are lysed with detergent and serial dilutions are performed. Serial dilutions and not diluted sample are grown in BHI-agar plates for 36-38 hours. At this time Colony-forming units (CFUs) can be counted.

## 12. Antibodies and reagents

Rapamycin, bafilomycin A1, 3-MA, cloroquine,  $\text{NH}_4\text{Cl}$ , 1400W and DPI were from Sigma-Aldrich and are illustrated in **Table 3.3**.

**Table 3.3. Table of Autophagy inhibitors.** Table of inhibitors used in experimental procedures. Concentration and function of experimental use are indicated.

Inhibitors	Concentraiton	Function
<b>1400W dihydrochloride</b>	10 $\mu\text{M}$	Specific inhibitor of inducible nitic oxide synthase (iNOS)
<b>3-methyladenine (3-MA)</b>	0,5 mM	Inhibitor of the formation of phagosome by inhibition of class III phosphatidylinositol-3 phosphate (PI3P)
<b>Bafilomycin A1</b>	20 nM	Inhibits vacuolar H <sup>+</sup> ATPase dampening the fusion of autophagosome with lysosome
<b>Cloroquine diphosphate</b>	50 $\mu\text{M}$	Cystein proteases inhibitor which impairs acidification of lysosome and late endosomes
<b>Diphenyleneiodonium chloride (DPI)</b>	5 $\mu\text{M}$	Inhibitor of the NADPH oxidase
<b><math>\text{NH}_4\text{Cl}</math></b>	50 mM	Inhibit the acidification of lysosome and late endosomes
<b>Rapamycin</b>	4 $\mu\text{M}$	Inhibitor of mTOR. Increase autophagy flux

Poly-L-lysine (PLL) was purchased from Sigma. Phalloidin-Alexa488 and 647 were from BD Biosciences. Zenon Alexa Fluor 488 rabbit IgG labelling kit, DAPI and Prolong Gold anti-fade mounting medium were from Thermofisher Scientific. Anti-human CD3 antibody (T3b hybridoma) was grown in Dr. F. Sánchez-Madrid laboratory (Hospital Universitario de la Princesa, HUP, Madrid) [158].

TLR agonists used for the activation of DCs are listed in **Table 3.4**, indicating their specific TLR activation, the used concentration and the brand.

**Table 3.4. TLR agonists.** Table of TLR agonists used in experimental procedures disclosed by target TLR, concentration and brand.

Agonist	TLR activation	Concentration	Brand
<i>Lm</i>	various	MOI=10	EGD strain
Heat killed <i>Lm</i> (HKLM)	various	MOI=50	InvivoGen
Heat killed <i>S. Typhimurium</i> (HKST)	various	MOI=50	InvivoGen
Palmitoyl-2-cysteine-serine-(lysine)-4 (Pam2GSK4)	TLR-2/6	10 µg/ml	InvivoGen
Palmitoyl-3-cysteine-serine-(lysine)-4 (Pam3GSK4)	TLR-1/2	10 µg/ml	InvivoGen
Polyinosinic-polycytidylic acid (Poly(I:C)) HMW	TLR-3	20 µg/ml	InvivoGen
Lipopolysaccharide (LPS)	TLR-4	1 mg/ml	Sigma-Aldrich
Flagellin	TLR-5	100 µg/ml	InvivoGen
Imiquimod	TLR-7	1 mg/ml	InvivoGen

### 13. Gene overexpression and silencing

HEK293 cells were co-transfected with plasmids encoding human MyD88 fused to the influenza hemmagglutinin (HA)-tag (Addgene plasmid #12287) together with plasmids encoding *HDAC6-WT* or double mutant deacetylase domain DD (mutated human HDAC6-H216A/H611A) fused to the enhanced Green Fluorescent protein (eGFP) tag (*HDAC6-WT* and *HDAC6-DD* previously described [220]). When indicated, cells were co-transfected with the appropriate small harping RNA plasmid pLVX-IRES-ZsGreen1, where *shHDAC6-2049* (TRCN0000004842) was cloned between BamH1 and EcoR1 sites. Cells were transfected using Lipofectamine 2000 (Invitrogen). Experiments were performed 24 h after transfection.

### 14. RNA isolation

RNA from mouse GM-CSF- derived DCs was isolated with the QIAGEN RNeasy Kit (Qiagen). Residual DNA contamination was removed with the Turbo DNA-free Kit (Ambion). RNA purity and concentration was analysed in a Nanodrop-1000 Spectrophotometer (Thermo Scientific). RNA integrity was assessed by etidium bromide labelling on a 1.5% agarose gel.

### 15. Reverse transcription and quantitative real-time-PCR

Total RNA (1-2 µg) was reverse transcribed to cDNA with a Reverse Transcription Kit (Applied Biosystems). Quantitative PCR was then performed in an AB7900-384 thermocycler (Applied Biosystem) using SYBR Green master mix (Applied Biosystems, Warrington, UK) as the reporter. PCR reactions were performed by triplicate in 10 µl volumes in 384-well plates. Expression levels of target genes were normalized to the expression of housekeeping genes  $\beta$ -actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH),  $\beta$ 2-microglobulin and Ywhaz (tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein,  $\zeta$ ) for presentation of relative mRNA levels. Data were analysed with Biogazelle qBasePlus version 2.3 (Biogazelle) and graphs are represented as a normalized expression scaled to average of all samples. Gene-specific primers used are listed in Table 3.5.

**Table 3.5. qPCR primers.** Table of qPCR primers used in experimental procedures disclosed by gene name and sequence 5'-3'. (Fw: Forward, Rv: Reverse).

Gene	Sequence (5'-3')	
<b>Atg12</b>	<b>Fw:</b> AACAAAGAAATGGGCTGTGGAGCG	<b>Rv:</b> TTCCGAGGCCACCAGTTTAAGGAA
<b>Atg2</b>	<b>Fw:</b> CCACCTCTGCAAATCGGCA	<b>Rv:</b> CCAGTTGCTCTGATACCTCCA
<b>Atg5</b>	<b>Fw:</b> GACAAAGATGTGCTTCGAGATGTG	<b>Rv:</b> GTAGCTCAGATGCTCGCTCAG
<b>Atg7</b>	<b>Fw:</b> ATGCCAGGACACCCTGTGAACCTC	<b>Rv:</b> ACATCATTGCAGAAGTAGCAGCCA
<b>Beclin-1</b>	<b>Fw:</b> GGCCAATAAGATGGGTCTGA	<b>Rv:</b> CACTGCCTCCAGTGTCTTCA
<b>CXCL10</b>	<b>Fw:</b> CCAAGTGCTGCCGTCATTTTC	<b>Rv:</b> TCCCTATGGCCCTCATTCTCA
<b>CXCL5</b>	<b>Fw:</b> TCGGTTGTGTTTGCTTAACCG	<b>Rv:</b> CTTCCACCGTAGGGCACTG
<b>CXCR1</b>	<b>Fw:</b> TCTGGACTAATCTGAGGGTG	<b>Rv:</b> GCCTGTTGGTTATTGGAACCTCTC
<b>GAPDH</b>	<b>Fw:</b> AGCTTGTCAACCGGGAAG	<b>Rv:</b> TTTGATGTTAGTGGGGTCTCG
<b>HDAC1</b>	<b>Fw:</b> CAGTCATGTCCAAAGTAAT	<b>Rv:</b> GTCCTTTGATGGTCAGATTG
<b>HDAC10</b>	<b>Fw:</b> ACAGCCACTCGACTGCTCT	<b>Rv:</b> GATGCCTCACAAGCTGACAAA
<b>HDAC11</b>	<b>Fw:</b> GTGTACTACCCACGTTACAACA	<b>Rv:</b> GCTCGTTGAGATAGCGCCTC
<b>HDAC2</b>	<b>Fw:</b> TGAAATTCCCAATGAGTTGC	<b>Rv:</b> GTTCTGGTTTGTCTGTTTG
<b>HDAC3</b>	<b>Fw:</b> GTGACATGTATGAAGTTGGAG	<b>Rv:</b> AAAAGGTGCTTGTAACCTCTG
<b>HDAC4</b>	<b>Fw:</b> CTAAGTGGTATGGGAAGACAC	<b>Rv:</b> TTTCTAAGAGGGGAAGTCA
<b>HDAC5</b>	<b>Fw:</b> AAGACCTAGAAGAGGAGGAG	<b>Rv:</b> GCGAACAACCTTTTGTAACC
<b>HDAC6</b>	<b>Fw:</b> TCCACCGGCCAAGATTCTTC	<b>Rv:</b> CAGCACACTTCTTCCACCAC
<b>HDAC6b</b>	<b>Fw:</b> TCGCTGTCTCATCCTACCTGCT	<b>Rv:</b> GTCAAAGTTGGCACCTTCACGG
<b>HDAC6-Ex12</b>	<b>Fw:</b> CTGGCTAAGGGAGTCAGTGC	<b>Rv:</b> TAGCACGGCTTCTTCCACTT
<b>HDAC7</b>	<b>Fw:</b> GAACTCTTGAGCCCTTGACA	<b>Rv:</b> GGTGTGCTGCTACTACTGGG
<b>HDAC8</b>	<b>Fw:</b> ACTATTGCCGGAGATCCAATGT	<b>Rv:</b> CCTCCTAAAATCAGAGTTGCCAG
<b>HDAC9</b>	<b>Fw:</b> GCGGTCCAGGTTAAACAGAA	<b>Rv:</b> GCCACCTCAAACACTCGCTT
<b>IFIT3</b>	<b>Fw:</b> GTGGACTGAGATTTCTGAACTGC	<b>Rv:</b> CAGAGATTCCCGGTTGACCT
<b>IFN-<math>\beta</math></b>	<b>Fw:</b> TCAGAATGAGTGGTGGTTGC	<b>Rv:</b> GACCTTTCAAATGCAGTAGATTCA
<b>IL12p40</b>	<b>Fw:</b> GGAAGCACGGCAGCAGAAT	<b>Rv:</b> AACTTGAGGGAGAAGTAGGAATGG
<b>IL-1<math>\beta</math></b>	<b>Fw:</b> GCAACTGTTCTGAACTCAACT	<b>Rv:</b> ATCTTTGGGGTCCGTCAACT
<b>ISG15</b>	<b>Fw:</b> GGTGTCCGTGACTAACTCCAT	<b>Rv:</b> TGAAAAGGGTAAGACCGTCTT
<b>LAMP-1</b>	<b>Fw:</b> AGCATACCGGTGTGTCACTG	<b>Rv:</b> GTTGGGGAAGGTCCATCCTG
<b>LAMP-2a</b>	<b>Fw:</b> TGGCTAATGGCTCAGCTTTC	<b>Rv:</b> ATGGGCACAAGGAAGTTGTC
<b>LC3A</b>	<b>Fw:</b> TTGGTCAAGATCATCCGGC	<b>Rv:</b> GCTCACCATGCTGTGCTGG
<b>LC3B</b>	<b>Fw:</b> CCCACCAAGATCCCAGTGAT	<b>Rv:</b> CCAGGAACCTGGTCTTGTTCA
<b>Mx1</b>	<b>Fw:</b> GACCATAGGGGTCTTGACCAA	<b>Rv:</b> AGACTTGCTCTTTCTGAAAAGCC
<b>p62</b>	<b>Fw:</b> ATGTGGAACATGGAGGGAAGA	<b>Rv:</b> GGAGTTCACCTGTAGATGGGT
<b>PanIFN-<math>\alpha</math></b>	<b>Fw:</b> CCTGAGARAGAAGAAACACAGCC	<b>Rv:</b> GGCTCTCCAGAYTTCTGCTCT
<b>Sirtuin-1</b>	<b>Fw:</b> ATGACGCTGTGGCAGATTGTT	<b>Rv:</b> CCGCAAGGCGAGCATAGAT
<b>Sirtuin-2</b>	<b>Fw:</b> ATCGTGTGTTTCGGTGAGAACC	<b>Rv:</b> TTCCTTGTTAATGAGCAGCCG
<b>Sirtuin-3</b>	<b>Fw:</b> CTTCCGCTAAACTTCTCCCG	<b>Rv:</b> ACAGAGGGATATGGGCCTTCT
<b>Sirtuin-4</b>	<b>Fw:</b> TCTGACGATTTGGCTTGCTT	<b>Rv:</b> TTCAGAGTTGGAGCGGCATT
<b>Sirtuin-5</b>	<b>Fw:</b> CTCCGGGCGGATTCATTTCC	<b>Rv:</b> GCGTTCGCAAAACACTTCCG
<b>Sirtuin-6</b>	<b>Fw:</b> ATGTCGGTGAATTATGCAGCA	<b>Rv:</b> GCTGGAGGACTGCCACATTA
<b>Sirtuin-7</b>	<b>Fw:</b> AGCATCACCCGTTTGCATGA	<b>Rv:</b> GGCAGTACGCTCAGTCACAT
<b>TLR-1</b>	<b>Fw:</b> TGAGGGTCTGATAATGTCCTAC	<b>Rv:</b> AGAGGTCAAATGCTTGAGGC
<b>TLR-2</b>	<b>Fw:</b> GCAAACGCTGTTCTGCTCAG	<b>Rv:</b> AGGCGTCTCCCTCTATTGTATT
<b>TLR-6</b>	<b>Fw:</b> TGAGCCAAGACAGAAAACCCA	<b>Rv:</b> GGGACATGAGTAAGGTTCTGTT
<b>TNF-<math>\alpha</math></b>	<b>Fw:</b> CCCTTCTCCGATGGCTAC	<b>Rv:</b> CGCTCCTTCTTGTCTGG
<b>Ywhaz</b>	<b>Fw:</b> CGTTGTAGGAGCCCGTAGGTCAT	<b>Rv:</b> TCTGGTTGCGAAGCATTGGG
<b><math>\beta</math>2-M</b>	<b>Fw:</b> TTCTGGTGCTGTCTCACTGA	<b>Rv:</b> CAGTATGTTGCGCTTCCCATTC
<b><math>\beta</math>-actin</b>	<b>Fw:</b> CAGAAGGAGATTACTGCTCTGGCT	<b>Rv:</b> TACTCTGCTTGCTGATCCACATC

Primers HDAC6-Ex12 were designed to amplify the exon 12 of *Hdac6* gene and were included in experiments as a control to check *Hdac6*<sup>-/-</sup> mice (deletion of the fragment corresponding to exons 10-13). Amplification after cycle 30-35 was observed in *Hdac6*<sup>-/-</sup> DCs, indicating the absence of exon 12. HDAC6 and HDAC6b primer pairs were designed to amplify exons before 10-13 fragment, so they were used as positive controls of amplification in *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> GM-CSF DCs.

## 16. Soluble embryonic alkaline phosphatase (SEAP)-NF-κB detection

Transfected HEK-Blue hTLR2 cells (50.000 per well) with different HDAC6 constructions were placed in bottom p96 well plates resuspended in HEK-Blue Detection medium (Invivogen) without stimulus (negative control) and with TLR-2 agonists (HKLM stimulus, MOI=10). After 8-12 h of incubation, SEAP activity was measured by optical density at 620 nm with a microplate reader. To calculate the NF-κB induction, the signal obtained from each mutant condition without stimuli (background) was depleted of the signal of each condition of activation with Pam2GSK4, Pam3GSK4 or HKLM.

## 17. ELISAs and nitrite measurement

Cytokine and Nitric Oxide (NO) production was analysed in the supernatants of BMDC cultures at 6, 12 and 24 h after stimulation with *Lm*, heat-killed *Listeria monocytogenes* (HKLM), heat-killed *Salmonella* Typhimurium (HKST), Pam3CSK4, Flagellin, Imiquimod, polyinosinic-polycytidylic acid (Poly(I:C)) (InvivoGen, San Diego, CA), or LPS from *Escherichia coli* (Sigma-Aldrich). TNF-α and IL12p70 were analysed with OptEIA Enzyme-Linked Immunosorbent Assay (ELISA) kits (BD Biosciences, San Diego, CA), IL-1β and IL-6 with the mouse ELISA Ready-SET-Go! kit from eBioscience (Affymetrix, San Diego, CA) and Interferon-β was measured with Legend max mouse IFN-β ELISA kit (Biolegend). The detection was based on colorimetric quantification of absorbance at 450 nm, corrected with subtraction at 570 nm measured in a microplate reader (Bio-Rad Model 550). Nitric oxide (NO) was estimated from the nitrite concentration measured with a Griess reagent kit at 548 nm (Molecular Probes/Life Technologies, Thermo Fisher Scientific). Results were expressed as the means of duplicate wells.

## 18. Immunoblotting

Total cell extracts from GM-CSF DCs stimulated with *Lm*, HKLM or the indicated TLR ligands for the indicated times were prepared in lysis buffer (0.5% Triton X100, 25 mM Tris-HCl pH 7.5, 0.5 mM EGTA, 0.5 mM EDTA, 25 mM NaF, 0.5 sodium glycerol-phosphate, 2.5 mM pyrophosphate, 0.135 M saccharose) with a cocktail of protease and phosphatase inhibitors (Roche). Cell lysates were cleared of nuclei by centrifugation at 15,000 g for 15 min. Protein extracts were quantified using Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad) and sample buffer Laemmli were added to protein lysates.



Protein extracts were separated by 8-15% SDS-PAGE using acrylamide/bisacrylamide 29:1 reactive (Biorad) following the Laemmli method with Tris-glycine buffer. Proteins were transferred to a PVDF membrane (Biorad) or 0.45 µm pore-size nitrocellulose membranes (Biorad). Membranes were blocked with TBS-Tween20 5% BSA. Proteins were visualized with LAS-3000 using Luminata Forte and Luminata Crescendo Western horseradich peroxidase (HRP) substrate (Millipore) after membrane incubation with specific antibodies (**Table 3.6**) and peroxidase-conjugated secondary antibodies (using 5 µg ml<sup>-1</sup> approximately) (**Table 3.7**). Band intensities were quantified using Image Gauge software (Fuji Photo Film, Co., Ltd) and results are expressed relative to loading controls. For quantification of western-blot, phosphorylated/total ratios were divided by loading control signal. Non-infection (NI) time was considered as 100%, and following times were relativized to it.

**Table 3.6. Primary antibodies for WB.** Table of antibodies used in WB experimental procedures disclosed by reference, brand, host and dilution (WB: Western blot).

Primary Antibodies	Reference	Brand	Host	Dilution
<b>AKT</b>	#9272	Cell Signaling	rabbit	WB (1:1000)
<b>ERK1/2</b>	#9102	Cell Signaling	mouse	WB (1:1000)
<b>HDAC6</b>	C0226	Assay bioTech	rabbit	WB (1:500)
<b>iNOS</b>	# 2982S	Cell Signaling	rabbit	WB (1:500)
<b>LC3b</b>	# 2775S	Cell Signaling	rabbit	WB (1:500)
<b>MyD88</b>	D80F5 #4283	Cell Signaling	rabbit	WB (1:500)
<b>MyD88</b>	sc-11356 (HFL-296)	Santa Cruz	rabbit	WB (1:500)
<b>MyD88</b>	sc-136970 (B-1)	Santa Cruz	mouse	WB (1:500)
<b>MyD88</b>	MA5-15762 (2E9C2)	TermoFisher Scientific	mouse	WB (1:500)
<b>p62</b>	P0067	Sigma-Aldrich	rabbit	WB (1:1000)
<b>p70S6K</b>	#2703	Cell Signaling	rabbit	WB (1:1000)
<b>phAKT (Ser473)</b>	#9271	Cell Signaling	rabbit	WB (1:1000)
<b>phERK1/2 (Thr202/Thr204)</b>	#9101	Cell Signaling	rabbit	WB (1:1000)
<b>php70S6K</b>	#9205	Cell Signaling	rabbit	WB (1:1000)
<b>phS6 (Ser235/236)</b>	#2211	Cell Signaling	rabbit	WB (1:1000)
<b>S6</b>	#2717	Cell Signaling	rabbit	WB (1:1000)
<b>tubulin</b>	T9026 (DH1A)	Sigma-Aldrich	mouse	WB (1:2000)
<b>β-actin</b>	A2228 (AC-74)	Sigma-Aldrich	mouse	WB (1:2000)

**Table 3.7. Secondary antibodies for WB.** Table of antibodies used in WB experimental procedures disclosed by reference, brand, host and dilution (WB: Western blot).

Secondary Antibodies	Reference	Brand	Host	Dilution
<b>Goat anti-mouse-HRP</b>	#31430	ThermoFisher Scientific	goat	WB (1:5000)
<b>Goat anti-rabbit-HRP</b>	#31460	ThermoFisher Scientific	goat	WB (1:5000)

Membrane strippings were performed using BlotFresh Western Blot Stripping reagent (SigmaGen Laboratories), followed by several Tris-buffered saline (TBS)-Tween 1% washes and TBS-Bovine serum albumin (BSA) 5% blockade previously to other primary antibody incubation.



## 19. Immunoprecipitation of MyD88 and HDAC6 proteins

### 19.1. From human monocyte-derived dendritic cells (moDCs)

Human moDCs ( $1 \times 10^7$  per condition) were lysed (10 mM Tris pH 7.4, 150 mM NaCl, 5% glycerol, 1mM EDTA, 1mM MgCl<sub>2</sub>, 1mM CaCl<sub>2</sub>, 1% CHAPS (Sigma) and protease and phosphatase inhibitors (Roche)) for 1 h at 4 °C. Lysates were incubated for pre-clearing with pre-washed Protein G Dynabeads (Invitrogen; 50 µl per condition; 2 h, 4 °C). Pre-cleared lysates were incubated with 6 µg rabbit anti-MyD88 antibody (Cell Signaling) or 6 µg rabbit anti-HDAC6 antibody (Assay BioTech) per condition O/N at 4 °C. Similar µg of control isotype antibody for rabbit were used. Fifty microlitres of Dynabeads per condition were washed three times in wash buffer (10 mM Tris pH 7.4, 150 mM NaCl, 5% glycerol, 1mM EDTA, 1mM MgCl<sub>2</sub>, 1mM CaCl<sub>2</sub>, 0.1% CHAPS) and added to antibody-conjugated lysates for 2 h 4C. Antibody-conjugated Dynabeads were washed six times with wash buffer and transferred to clean tubes.

### 19.2. From HEK cell lines

HEK293T cells or HEK-Blue hTLR2 ( $1 \times 10^7$  per condition) were lysed (25 mM Tris pH 8, 150 mM NaCl, 0.5% NP-40 and protease and phosphatase inhibitors) and incubated for pre-clearing with pre-washed Protein G Dynabeads (Invitrogen; 50 µl per condition; 3 h, 4 °C). Fifty µg of Dynabeads per condition were washed three times in wash buffer (25 mM Tris pH 8, 150 mM NaCl, 0.05% NP-40) and re-suspended in 600 µl of wash buffer containing 1-2 µg mouse anti-HA antibody (Roche) per condition and incubated 3 h at 4 °C. Similar µg of control isotype antibody for mouse were used. Pre-cleared lysates were incubated with antibody-conjugated Dynabeads (O/N, 4 °C). Antibody-conjugated Dynabeads were washed six times with lysis buffer and transferred to clean tubes. Then, were washed twice with wash buffer. Protein loading buffer was added, samples were boiled at 95 °C for 5 min and processed for immunoblotting.

Antibodies used for IP approach are listed in **Table 3.8** indicating the reference, brand, host and concentration of use.

**Table 3.8. Primary antibodies for IP.** Table of antibodies used in IP experimental procedures disclosed by reference, brand, host and dilution (IP: Immunoprecipitation).

Primary Antibodies	Reference	Brand	Host	Dilution
Anti-HA	000000011583816001 (12CA5)	Roche (Sigma-Aldrich)	mouse	IP (5 µg/point)
Anti-HA	A190-108A	Bethyl	rabbit	IP (5 µg/point)
Control isotype of mouse	sc-2025	Santa Cruz	rat	IP (1-5 µg/point)
Control isotype of rabbit	sc-2027	Santa Cruz	rabbit	IP (5 µg/point)

## 20. Flow cytometry

Cells were stained in ice-cold PBS containing FBS (0.5%) and EDTA (5 mM) using appropriate antibody-fluorophore conjugates. Multiparameter analysis was performed on a FACSCANTO II flow cytometer (BD Biosciences) and analysed with FlowJo software (Tree Star). Prior to fixing, cells were resuspended in PBS/0.5% BSA/5 mM EDTA solution containing yellow fluorescent reactive dye to exclude dead cells (Life Technologies). For intracellular staining, cells were fixed and permeabilized using the CytoFix/Cytoperm kit (BD). Human cells were blocked with human gamma-globulin from human blood (Sigma), whereas mouse cells were blocked using Fc-Block (CD16/32) (Tombo biociences). Complete primary and secondary antibody used for FC are listed in **Table 3.9** and **Table 3.10**.

**Table 3.9. Primary antibodies for FC.** Table of antibodies used in FC experimental procedures disclosed by reference, brand, host and dilution (FC: Flow cytometry).

Primary Antibodies	Reference	Brand	Host	Dilution
<b>B220-PerCP-cyanine5.5</b>	65-0452-U100 (RA 3-6B2)	Tombo biociences	rat	FC (1:200)
<b>CD11b-FITC</b>	553310 (M1/70)	BD Pharmingen	rat	FC (1:200)
<b>CD11c-PE</b>	557401 (HL3)	BD Pharmingen	hamster	FC (1:200)
<b>CD11c-PE</b>	555392 (B-ly6)	BD biociences	mouse	FC (1:200)
<b>CD11c-PeCy7</b>	558079 (HL3)	BD Pharmingen	hamster	FC (1:200)
<b>CD11c-PeCy7</b>	55079	BD Pharmingen	hamster	FC (1:200)
<b>CD14-FITC</b>	14F-100T (47-3D6)	Immunostep	mouse	FC (1:200)
<b>CD16/32 Fc-block</b>	(clone 2.4G)	BD Pharmingen	rat	FC (1:200)
<b>CD19-VioletFluor450</b>	75-0193-U100 (1D3)	Tombo biociences	rat	FC (1:200)
<b>CD24-PE</b>	12-0241-82 (30-F1)	ebiocience	rat	FC (1:200)
<b>CD3e</b>	T3b hybridoma	[158]	mouse	FC (5 µg/ml)
<b>CD3-VioletFluor450</b>	75-0032-U100 (17A2)	Tombo biociences	rat	FC (1:200)
<b>CD40-APC</b>	558695	BD Pharmingen	rat	FC (1:200)
<b>CD86-biotinylated</b>	553690 (GL1)	BD Pharmingen	rat	FC (1:200)
<b>CD8-APC</b>	558079 (HL3)	BD Pharmingen	rat	FC (1:200)
<b>DC-SIGN (CD209)</b>	sc-59157 (MR-1)	Santa Cruz	mouse	FC (1:200)
<b>DX5 (CD49b)-V450</b>	561638	BD biociences	rat	FC (1:200)
<b>Gr-1-APC</b>	553129 (RB6-8C5)	BD Pharmingen	rat	FC (1:200)
<b>HLA-DR-PerCP</b>	560652 (G46-6)	BD biociences	mouse	FC (1:200)
<b>Lm</b>	0400-0030	AbDSerotec	rabbit	FC (1:200)
<b>Ly6C-PerCP-Cy5.5</b>	560525	Becton Dickinson	rat	FC (1:200)
<b>Ly6G-PE</b>	551461 (1A8)	BD biociences	rat	FC (1:200)
<b>MHC-II I-A/I-E-APC</b>	17-5321-81 (145/1/4.15.2)	ebiocience	rat	FC (1:200)
<b>MHC-II I-A/I-E-FITC</b>	553623 (2G9)	BD Pharmingen	rat	FC (1:500)
<b>p62</b>	P0067	Sigma-Aldrich	rabbit	FC (1:200)

**Table 3.10. Secondary antibodies for FC.** Table of antibodies used in FC experimental procedures disclosed by reference, brand, host and dilution (FC: Flow cytometry).

Secondary Antibodies	Reference	Brand	Host	Dilution
<b>Chicken anti-mouse 647</b>	A-21463	Life technologies	chicken	FC (1:500)
<b>Chicken anti-rabbit 647</b>	A-21443	Life technologies	chicken	FC (1:500)
<b>Streptavidin-APC</b>	554067	BD Pharmingen	-	FC (1:300)
<b>Streptavidin-BV421</b>	563259	BD biociences	-	FC (1:300)

## 21. Fluorescence confocal microscopy

For immunofluorescence assays, cells were plated onto slides coated with poly-L-lysine (50  $\mu\text{g ml}^{-1}$ ) and incubated for 1 h at 37°C. Infection experiments were carried out at the indicated times. Cells were then fixed, blocked and stained with the indicated primary antibodies (5  $\mu\text{g ml}^{-1}$ ) (Table 3.11) followed by Alexa488- or Rhodamine Red X-labelled secondary antibodies highly cross-adsorbed (Hx)(5  $\mu\text{g ml}^{-1}$ ) (Table 3.11 and Table 3.10).

**Table 3.11. Primary antibodies for IF.** Table of antibodies used in IF experimental procedures disclosed by reference, brand, host and dilution (IF: Immunofluorescence).

Primary Antibodies	Reference	Brand	Host	Dilution
Acetylated cortactin	09-881	Millipore	rabbit	IF (1:100)
p62	P0067	Sigma-Aldrich	rabbit	IF (1:200)
K63 polyUb chain	05-1308 (clone Apu3)	Merk-Millipore	rabbit	IF (1:100)

**Table 3.12. Secondary antibodies for IF.** Table of antibodies used in IF experimental procedures disclosed by reference, brand, host and dilution (IF: Immunofluorescence).

Secondary Antibodies	Reference	Brand	Host	Dilution
Goat anti-rabbit A488 Hx	A-11008	Thermofisher Scientific	goat	IF (1:500)
Goat anti-rabbit Rhodamin Red-X Hx	111-295-144	Jackson	goat	IF (1:500)

Samples were examined under a Leica SP5 confocal microscope (Leica) fitted with a 63X objective. Images were acquired with sequential xyz acquisition mode scans with laser ranges of 418-473 nm for DAPI, 502-548 nm for Alexa-488, 584-644 nm for Rhodamine Red-X and 737-779 nm for Alexa-647. Z-stacks of 2-5  $\mu\text{m}$  were obtained using a maximum z-step size of 0.3  $\mu\text{m}$ .

## 22. Imaris quantification

Images were processed and assembled using Image J 1.51p (Fiji). Confocal 3D images assembled with Imaris 7.7.2 (Bitplane) using the ImarisCell module. Every cell and its corresponding intracellular bacteria were calculated in each image. Surfaces corresponding to bacteria were used to calculate the maximal fluorescence intensity of the channels to co-localize with bacteria. Two-channel co-localization was quantified in at least 10 images per genotype, corresponding to 10 biological samples. Each image contains from 60 to 80 cells.

## 23. In-gel protein digestion

Proteins were in-gel digested using a previously described protocol [27]. Briefly, the coimmunoprecipitate was heated at 95 °C for 5 min, after which the magnetic beads were removed using a magnet. The resulting solution was added sample buffer and loaded in 0.5-cm-wide wells of an SDS-PAGE gel. The run was stopped as soon as the front entered into the resolving gel. The protein band was visualized by Coomassie Blue staining, excised, and digested overnight at 37 °C with 60 ng/ $\mu\text{l}$

sequencing-grade modified trypsin (Promega) at 10:1 protein:enzyme (w/w) ratio in 50 mM ammonium bicarbonate, pH 8.8, containing 10% acetonitrile. The resulting tryptic peptides were desalted onto C18 OMIX tips (Agilent), dried down and kept at -80 °C until further use.

## 24. Mass spectrometry

The resulting peptides were analyzed by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) on an Easy nLC-1000 nano-HPLC apparatus (Thermo Scientific, San Jose, CA, USA) coupled to a hybrid quadrupole-orbitrap mass spectrometer (Q Exactive HF, Thermo Scientific). The dried peptides were taken up in 0.1% (v/v) formic acid and then loaded onto a PepMap100 C18 LC pre-column (75 µm I.D., 2 cm, Thermo Scientific) and eluted on line onto an analytical NanoViper PepMap 100 C18 LC column (75 µm I.D., 50 cm, Thermo Scientific) with a continuous gradient consisting of 8-31% B in 240 min (B = 80% ACN, 0.1% formic acid) at 200 nL/min. Peptides were ionized using a Picotip emitter nanospray needle (New Objective). Each MS run consisted of enhanced FT-resolution spectra (120,000 resolution) in the 400–1,200 m/z range followed by data-dependent MS/MS spectra of the 20 most intense parent ions acquired along the chromatographic run. The AGC target value in the Orbitrap for the survey scan was set to 1,000,000. Fragmentation in the linear ion trap was performed at 30% normalized collision energy with a target value of 10,000 ions. The full target was set to 30,000, with 1 microscan and 50 ms injection time, and the dynamic exclusion was set to 0.5 min.

## 25. Peptide identification

For peptide identification the MS/MS spectra were searched with the Sequest algorithm implemented in Proteome Discoverer 1.4 (Thermo Scientific). Database searching against human protein sequences from the UniProt database (March 2017, 158,382 entries) was performed with the following parameters: trypsin digestion with 4 maximum missed cleavage sites; precursor and fragment mass tolerances of 800 ppm and 0.02 Da, respectively; Cys carbamidomethylation as static modifications; and Met oxidation and Lys acetylation as dynamic modifications. The results were analyzed using the probability ratio method [149] and a false discovery rate (FDR) for peptide identification was calculated based on the search results against a decoy database using the refined method [168].

## 26. Statistical analysis

Data were analysed with GraphPad prism software (La Jolla, CA) for normality (Kolmogorov-Smirnov test for small samples). Normal data were analysed by Student t-test, non-normal data by Mann-Whitney test, and grouped data by 2-tailed One-way ANOVA with a Bonferroni post-test. Graphs show

the distribution of every sample and the mean of all samples  $\pm$  the standard deviation of the mean (SEM).

For western blot quantification, the sample with the maximum signal was assigned a value of 100%, and signals in other samples were expressed as a percentage of this; significance was determined by a one-sample test.

Long-rank (Mantel-Cox) test and Cehan-Breslow-Wilcoxon test were used for the analysis of the Kaplan-Meier curve (survival curve).

Statistical significant differences were considered when  $p \leq 0.05$ , expressed as \*, whereas \*\* was used for differences  $p \leq 0.01$  and \*\*\* for differences  $p \leq 0.001$ . Non-significant differences were designed as ns ( $p > 0.05$ ).





Results



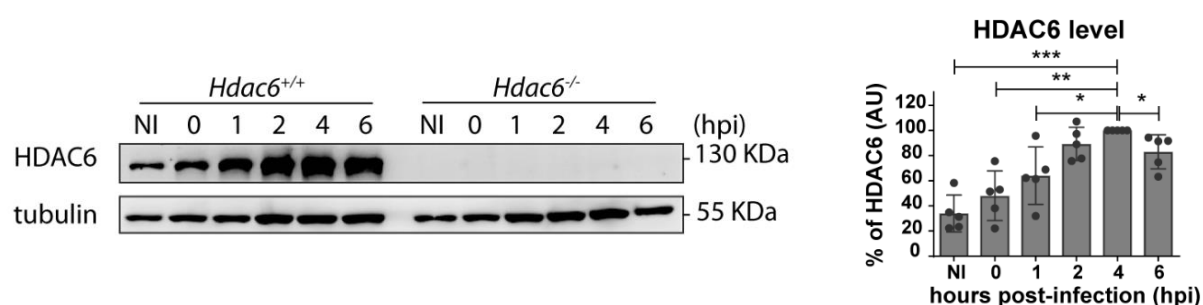


## 4. RESULTS

### 4.1. Analysis of HDAC6 contribution to cellular mechanisms that eliminate intracellular bacteria by phagocytic cells

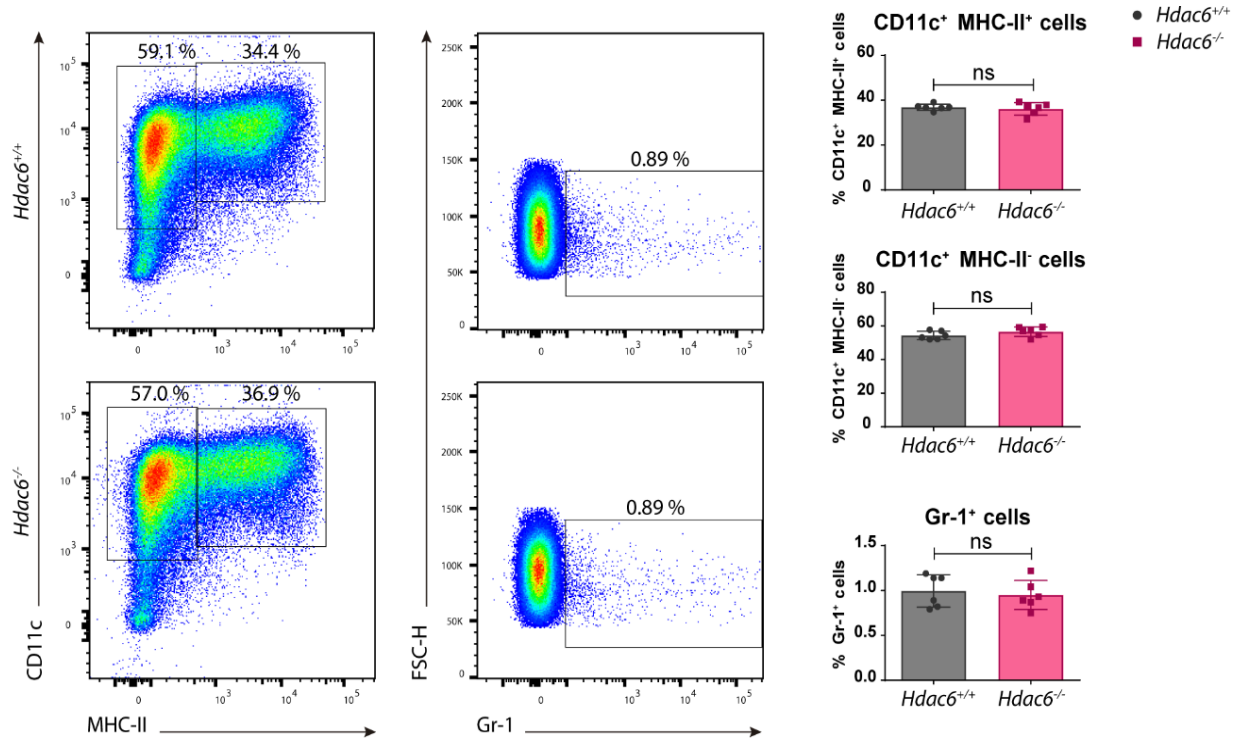
#### 4.1.1. Effect of HDAC6 absence in the intracellular pathogen clearance of dendritic cells

To assess the possible role of HDAC6 in innate immune responses during bacterial pathogenesis, we performed a time-course infection with *Lm* in bone marrow (BM)-derived granulocyte and monocyte colony-stimulating factor (GM-CSF)-derived dendritic cells (DCs) from *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> mice. Increasing levels of HDAC6 expression were detected in the *Hdac6*<sup>+/+</sup> DCs as the infection progressed, reaching a peak at 4 hpi (Figure 4.1).



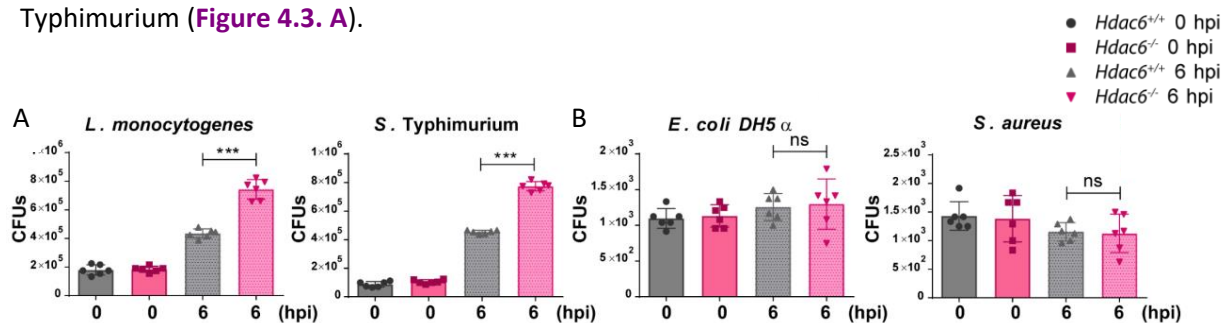
**Figure 4.1. HDAC6 protein induction during *Lm* infection.** *Left:* Western blot analysis of HDAC6 in a time-course of infection of GM-CSF DCs with *Lm*. Samples of non-infected (NI) cells and 0, 1, 2, 4 and 6 hpi cells are displayed. Tubulin was used as a loading control. *Right:* HDAC6 levels were quantified and are expressed relatively to its respective tubulin signal. Two-tailed t-test, \*\*\* $p \leq 0.001$ , \*\*  $p \leq 0.01$ , \*  $p \leq 0.05$ ;  $n=5$ .

However, GM-CSF-derived DC differentiation was not noticeably affected in the absence of HDAC6, since there are the same levels of CD11c+MHC-II+, CD11c+MHC-II- and Gr-1+ populations between *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> DCs at day 11 of *in vitro* differentiation (Figure 4.2).



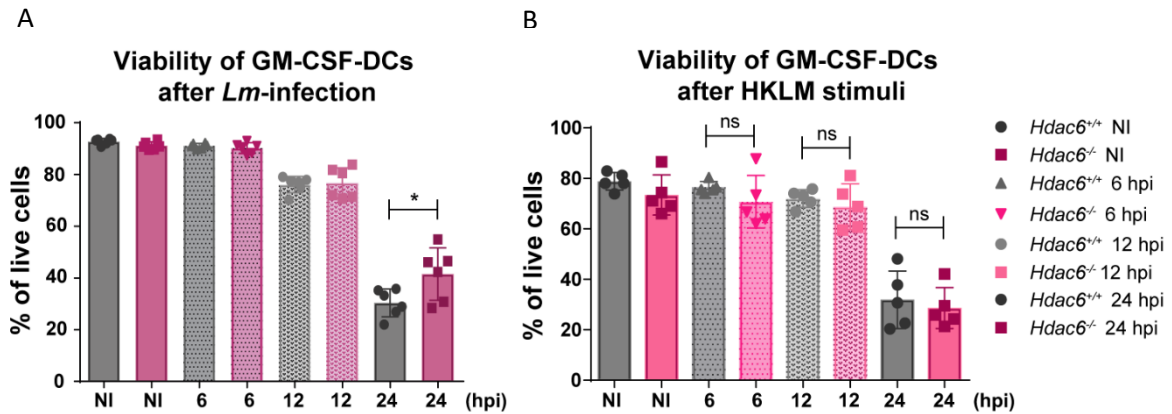
**Figure 4.2. Differentiation of GM-CSF-derived DCs at day 11 of culture.** Left: Dot-plots showing CD11c and MHC-II markers, with gating for CD11c<sup>+</sup>MHC-II<sup>+</sup> and CD11c<sup>+</sup>MHC-II<sup>-</sup> populations (percentages indicated). Middle: Dot-plots on differentiation day 11 showing FSC-H versus Gr-1, gating the Gr-1<sup>+</sup> population corresponding to neutrophil contamination in GM-CSF-derived DC cultures. Right: Charts show the percentages of CD11c<sup>+</sup>MHC-II<sup>+</sup>, CD11c<sup>+</sup>MHC-II<sup>-</sup> and Gr-1<sup>+</sup> populations. ns>0.05 non-significant; n=6.

Next, *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> BMDCs were infected for different times with *Gram*-negative bacteria (*Salmonella* Typhimurium and *Escherichia coli* DH5 $\alpha$ ) and *Gram*-positive bacteria (*Listeria monocytogenes* and *Staphylococcus aureus*) at a multiplicity of infection (MOI) of 10, with colony-formed units (CFUs) corresponding to intracellular live bacteria (Figure 4.3). Bacterial entry was similar in *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> DCs at 0 h post-infection (hpi), while bacterial proliferation, measured at 6 hpi, was significantly higher in *Hdac6*<sup>-/-</sup> DCs for both types of intracellular pathogens, *Lm* and *S. Typhimurium* (Figure 4.3. A).



**Figure 4.3. Intracellular bacteria entry and proliferation in GM-CSF-DCs infected with various types of bacteria.** CFUs obtained at 0 and 6 hpi from DCs infected with (A) intracellular pathogens (*L. monocytogenes*, *S. Typhimurium*), and (B) extracellular pathogens (*E. coli* DHD $\alpha$ , and *S. aureus*) at a MOI of 10. Data from 0 hpi are shown as bacteria entry control. Two-tailed t-test, \*\*\*p≤0.001, ns>0.05 non-significant; n=6.

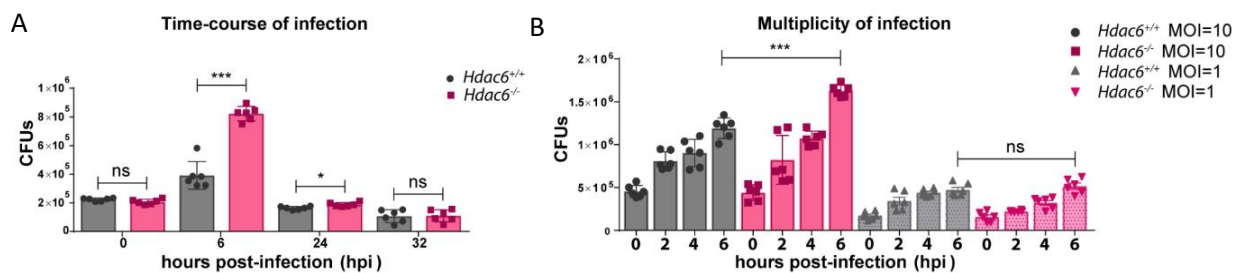
This was not due to differences in cell viability after 6 hpi (Figure 4.4. A). However, cell viability of *Hdac6*<sup>+/+</sup> DCs is lower than *Hdac6*<sup>-/-</sup> ones at 24 hpi (Figure 4.4. A). No-significant differences were observed between *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> DCs after the addition of Heat Killed *Lm* (HKLM) at any time (Figure 4.4. B). These data indicate a phenotype in cell viability only with live bacteria at 24 hpi. In contrast, no significant difference was observed in the proliferation of the non-intracellular pathogens *S. aureus* and *E. coli*, indicating that HDAC6 is an important component of cellular mechanisms for the clearance of intracellular pathogens (Figure 4.3. B).



**Figure 4.4. Viability of GM-CSF-DCs after *Lm* infection and HKLM stimuli at different times.** Percentage viability of GM-CSF-DCs before and after (A) the infection with *Lm* MOI=10 or (B) HKLM stimuli MOI=50 at different times, NI: non-infected, 6, 12 and 24 h. Two-tailed t-test, \*\*p<0.01, ns>0.05 non-significant; n=6.

#### 4.1.2. Measurement of *Lm* load in HDAC6-deficient myeloid cells

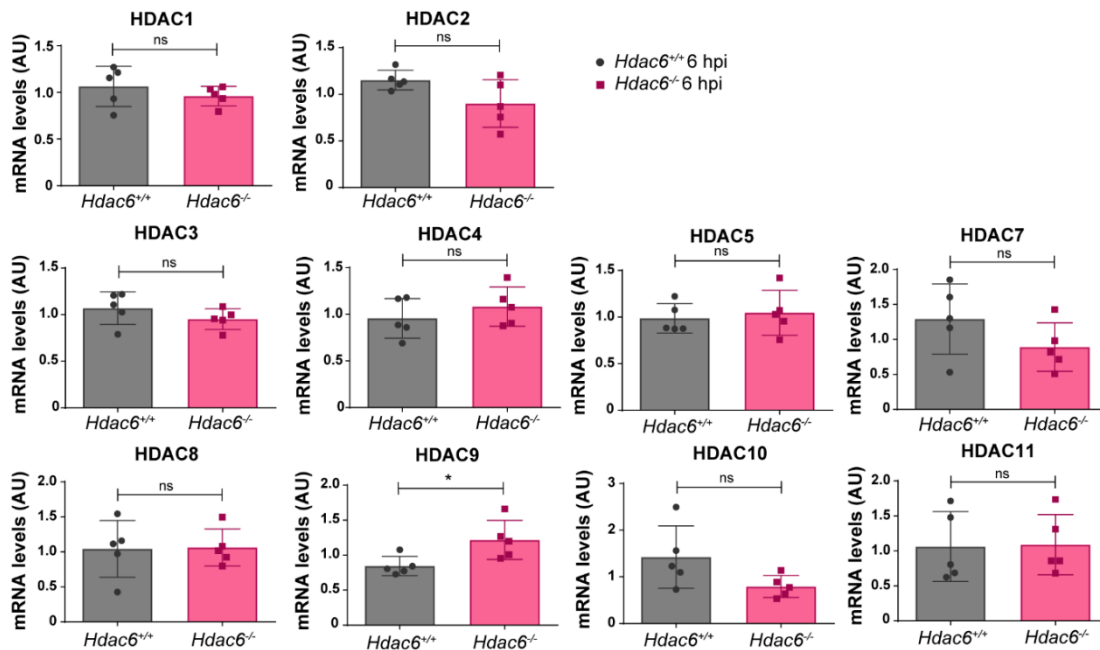
Time-course analysis showed that differences between *Lm* infection in *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> DCs CFUs peaked at 6 hpi and were sustained until 24 hpi (Figure 4.5. A). This effect was clearly observed at a MOI of 10, which did not affect cell viability (Figure 4.5. B and Figure 4.4).



**Figure 4.5. Time-course of *Lm* infection in GM-CSF-DCs and multiplicity of infection.** (A) CFUs of *Lm*-infected GM-CSF DCs obtained at 0, 6, 24 and 32 hpi with a MOI=10. \*\*\*p<0.001, \*p<0.05, ns>0.05 non-significant; n=6. (B) CFUs of *Lm*-infected DCs obtained at 0, 2, 4 and 6 hpi with a MOI=10 and 1. Two-tailed t-test, \*\*\*p<0.001, \*p<0.05, ns>0.05 non-significant; n=6.

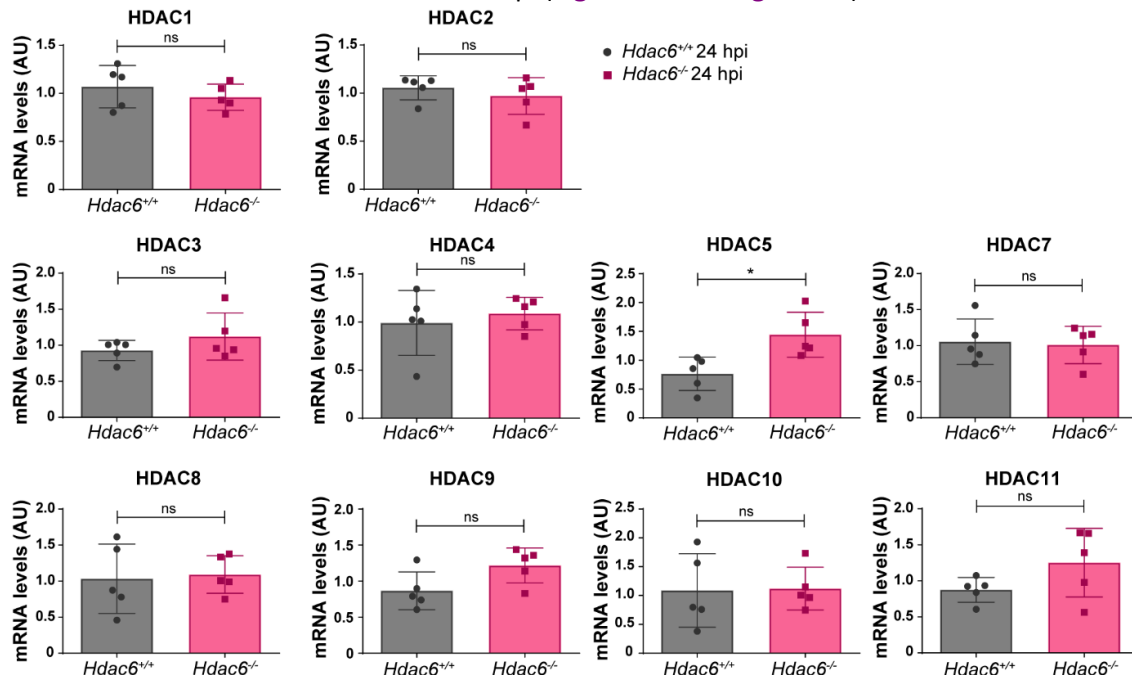
The lack of difference in bacterial load at 32 hpi between both genotypes is indicating that *Hdac6*<sup>-/-</sup> DCs do not succumb to the infection (Figure 4.5). This result suggests that other HDACs can be

compensating HDAC6 function during the elimination of intracellular *Lm* at long times of infection. To address this question, qPCRs of every mouse deacetylase, HDACs (1-5 and 7-11) and sirtuins (1-7), were performed in *Lm* infected DCs at 6 and 24 hpi (Figure 4.6, Figure 4.7, Figure 4.8 and Figure 4.9).



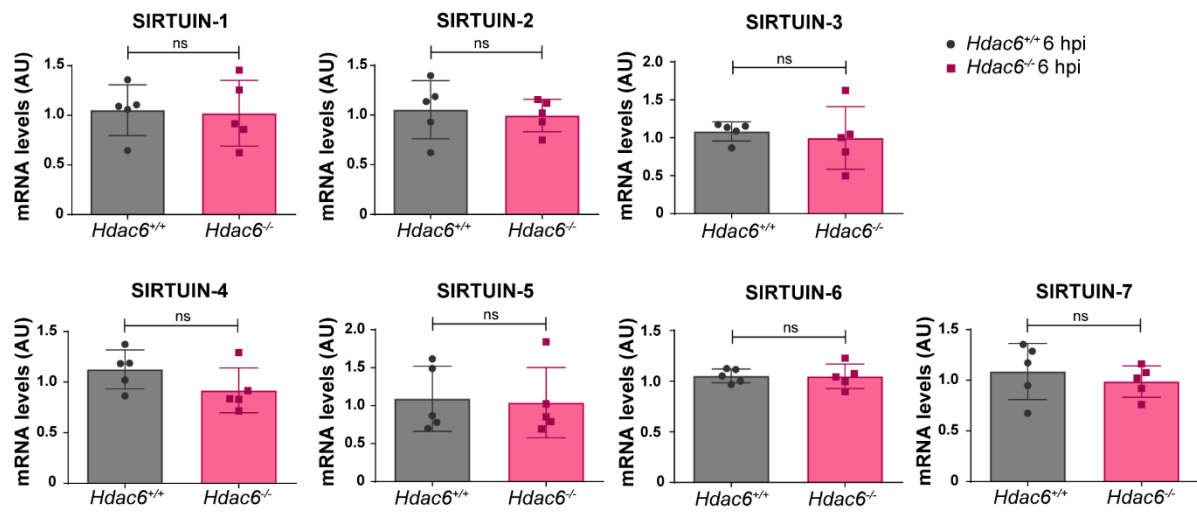
**Figure 4.6. Expression of HDACs in DCs after *Lm* infection at 6 hpi.** PCR analysis of HDACs (HDAC1, 2, 3, 4, 5, 7, 8, 9, 10 and 11) in *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> GM-CSF DCs infected with *Lm* at 6 hpi (AU: arbitrary units). Two-tailed t-test, \*  $p < 0.05$ , ns  $> 0.05$  non-significant;  $n = 5$ .

In this regard, higher expression levels of HDAC5 were detected in *Hdac6*<sup>-/-</sup> DCs at 24 hpi, whereas similar results were obtained for HDAC9 at 6 hpi (Figure 4.6 and Figure 4.7).

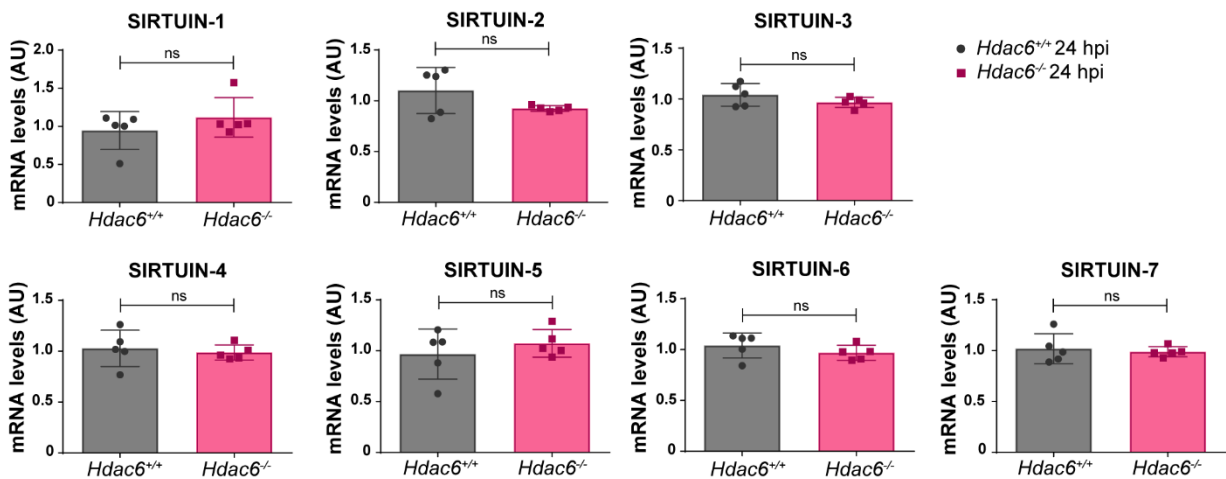


**Figure 4.7. Expression of HDACs in DCs after *Lm* infection at 24 hpi.** PCR analysis of HDACs (HDAC1, 2, 3, 4, 5, 7, 8, 9, 10 and 11) in *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> GM-CSF DCs infected with *Lm* at 24 hpi (AU: arbitrary units). Two-tailed t-test, \*  $p < 0.05$ , ns  $> 0.05$  non-significant;  $n = 5$ .

However, no significant differences were observed in sirtuins expression between *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> DCs at any time (Figure 4.8 and Figure 4.9).

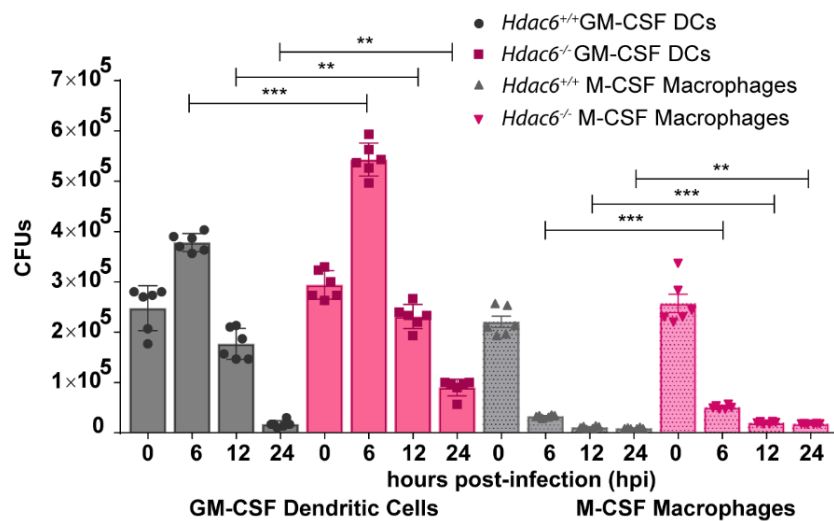


**Figure 4.8. Expression of Sirtuins in DCs after *Lm* infection at 6 hpi.** PCR analysis of Sirtuins (1, 2, 3, 4, 5, 6 and 7) in *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> GM-CSF DCs infected with *Lm* at 6 hpi (AU: arbitrary units). Two-tailed t-test, \*  $p \leq 0.05$ , ns > 0.05 non-significant; n=5.



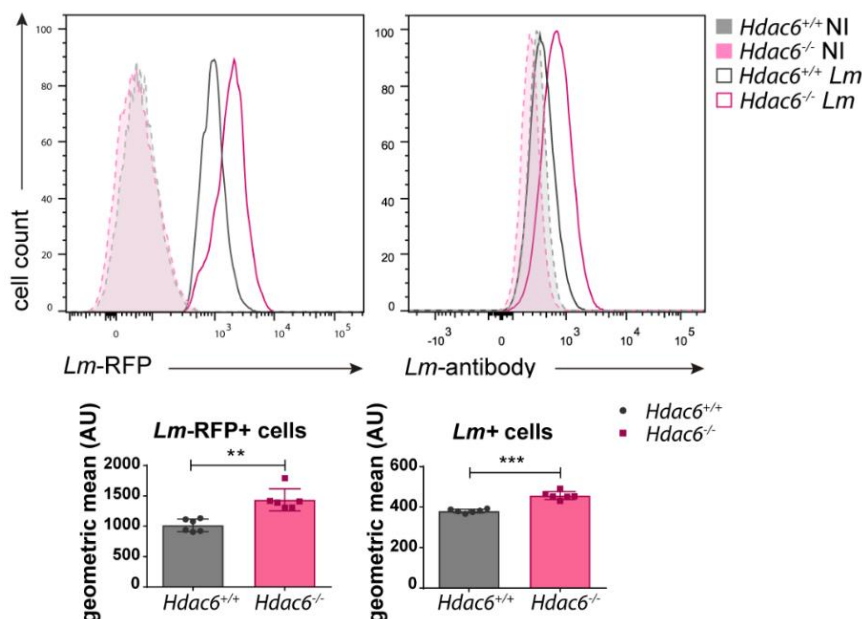
**Figure 4.9. Expression of Sirtuins in DCs after *Lm* infection at 24 hpi.** PCR analysis of Sirtuins (1, 2, 3, 4, 5, 6 and 7) in *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> GM-CSF DCs infected with *Lm* at 24 hpi (AU: arbitrary units). Two-tailed t-test, \*  $p \leq 0.05$ , ns > 0.05 non-significant; n=5.

A similar pattern was observed with macrophage colony-stimulating factor (M-CSF)-derived macrophages, demonstrating the lineage independence of the role of HDAC6 in bacterial clearance (Figure 4.10). Although the difference between *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> cells was observed in both macrophages and DCs, the clearance capacity of macrophages was ten-fold higher than that of DCs at 6 hpi (Figure 4.10).



**Figure 4.10. Comparison of intracellular bacteria in GM-CSF-derived DCs and M-CSF-derived macrophages over the time-course of *Lm*-infection.** CFUs of DCs and macrophages are shown at 0, 6, 12 and 24 hpi. In both cases *Hdac6*<sup>-/-</sup> cells display higher intracellular bacterial load. Two-tailed t-test, \*\*\*p ≤ 0.001, \*\* p ≤ 0.01, ns > 0.05 non-significant; n = 6.

Bacterial load was also determined by flow cytometry using two strategies: a specific antibody against *Lm*, and RFP-expressing bacteria. Both approaches showed that *Hdac6*-deficient DCs contained more bacteria at 6 hpi (Figure 4.11).

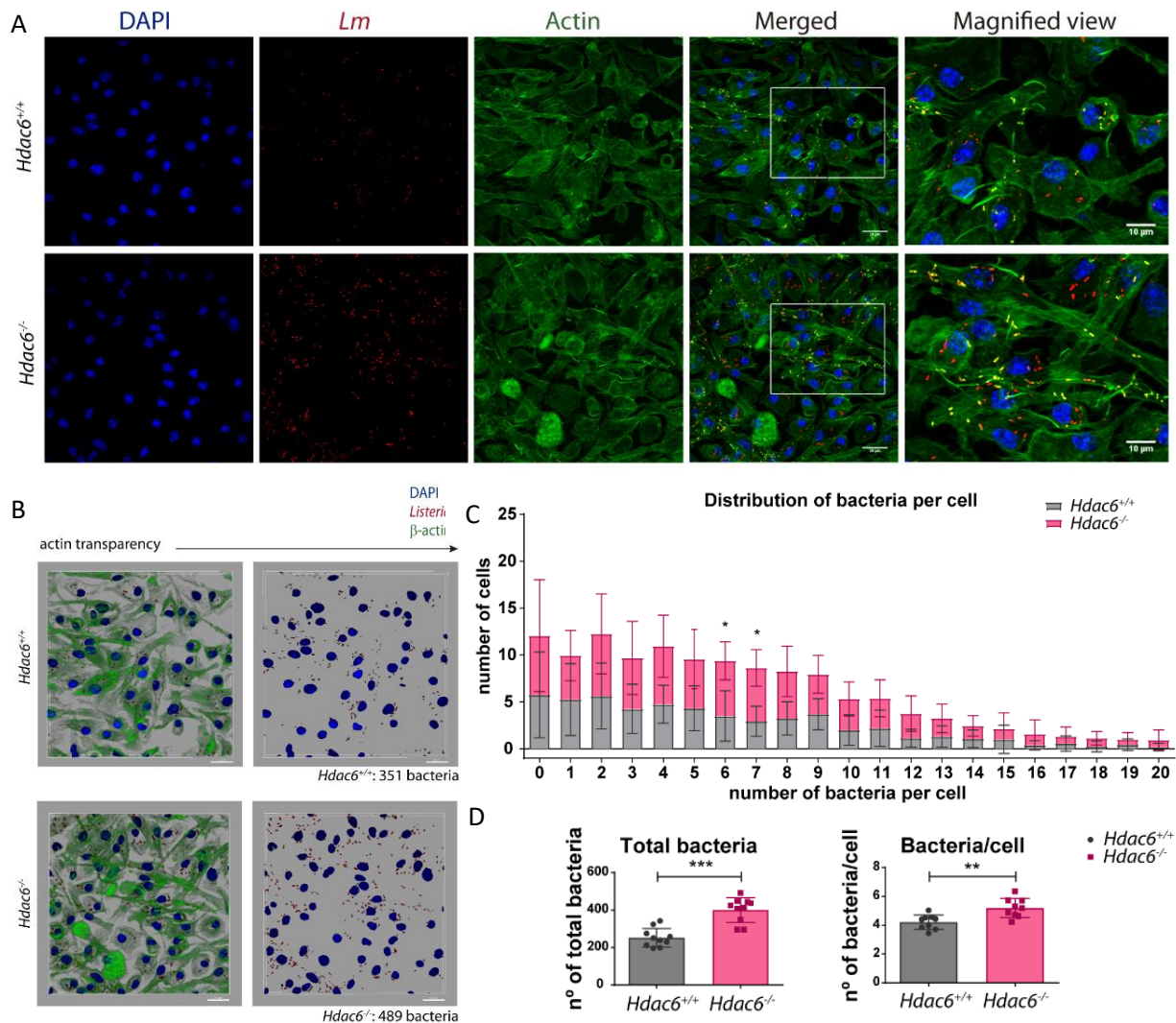


**Figure 4.11. GM-CSF DCs were infected with *Lm*-RFP or *Lm* for 6 h and the bacterial signal was determined by flow cytometry.** Top panel: The panel shows representative histograms of *Lm*-RFP and *Lm*-antibody signals. Bottom panel: Graphs show geometric mean of the *Lm* signals. Each dot represents the number of samples and the mean with SEM is indicated in colour. Two-tailed t-test, \*\*\*p ≤ 0.001, \*\* p ≤ 0.01; n = 6.

Higher numbers of bacteria in *Hdac6*<sup>-/-</sup> GM-CSF DCs were also detected by confocal fluorescence microscopy at 6 hpi (Figure 4.12). Some bacteria co-localized with filamentous actin, showing clear actin rockets (Figure 4.12. A and B). Image quantification confirmed that *Hdac6*<sup>-/-</sup> DCs contained more bacteria per cell and more total bacteria, remarking a higher percentage of cells hosting a large number of bacteria in *Hdac6*<sup>-/-</sup> cells (see distribution of bacteria per cell, 6-7) (Figure 4.12. C and D). ImarisCell



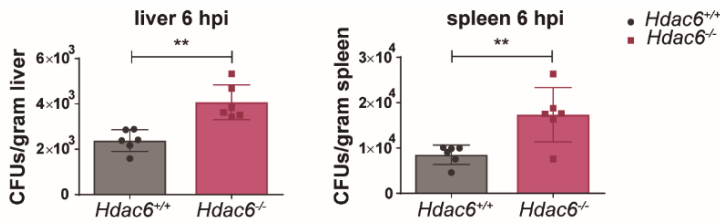
Module view of images showed the number of bacteria per cell using actin transparency to easily visualize individual bacteria (**Figure 4.12. B**).



**Figure 4.12. Confocal microscopy determination of bacterial load at 6 hpi.** (A) Maximum intensity z-projections of confocal microscopy images of *Lm*-infected *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> GM-CSF DCs at 6 hpi. The panel shows DAPI (blue), *Lm* (red),  $\beta$ -actin (green), merged views of three channels, and magnified views of the boxed areas from the merged view. Yellow indicates *Lm* and  $\beta$ -actin co-localization. Scale bars 20  $\mu$ m (main panels) and 10  $\mu$ m magnified views). (B) Confocal microscopy determination of bacterial load of **Figure 4.12.A**. Maximum intensity z-projections of confocal microscopy images of *Lm*-infected *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> DCs at 6 hpi. ImarisCell Module view of the number of nucleus and bacteria per cell. Actin transparency is used to visualize bacteria (number indicated on the right). Images show DAPI (blue), *Lm* (red),  $\beta$ -actin (green). Scale bars 20  $\mu$ m. (C) ImarisCell Module analysis of the number of cells and the number of bacteria per cell in all pictures (10 pictures per genotype). The graph shows the distribution of cells with a specific number of bacteria per cell. The number of cells with 6 and 7 bacteria differed significantly between the *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> genotypes. Two-way Anova, \*  $p < 0.05$ ;  $n = 10$ . (D) Graphs show statistical analysis of Imaris quantification of total bacteria and bacteria per cell in *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> DCs. Two-tailed t-test, \*\*\*  $p \leq 0.001$ , \*\*  $p \leq 0.01$ ;  $n = 10$ .

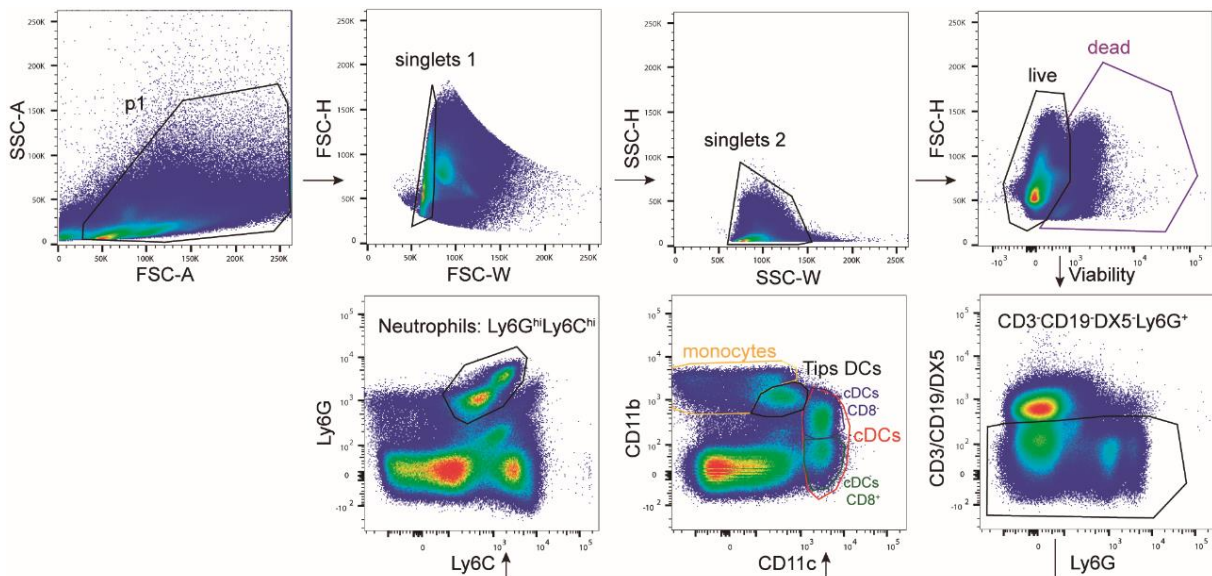
#### 4.1.3. Determination of *in vivo* bacterial burden

To ascertain whether *Hdac6*<sup>-/-</sup> cells display higher bacterial burden than *Hdac6*<sup>+/+</sup> cells *in vivo*, *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> mice were intravenously injected with *Lm* and total CFUs per gram of liver and spleen were determined at 6 hpi. In agreement with the higher numbers of *Lm* observed in GM-CSF-DCs and M-CSF-Macrophages, we observed increased bacterial titres in spleen and liver cell suspensions (**Figure 4.13**).



**Figure 4.13. Deficient intracellular bacteria clearance in target organs of *Hdac6*<sup>-/-</sup> mice.** Quantification of bacterial load in target organs (spleen and liver) at 6 hpi in *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> mice injected with a lethal dose of *Lm*. Bacterial load is expressed by CFUs per gram of liver (left graph) and per gram of spleen (right graph). Two-tailed t-test, \*\**p*≤0.01, *n*=6.

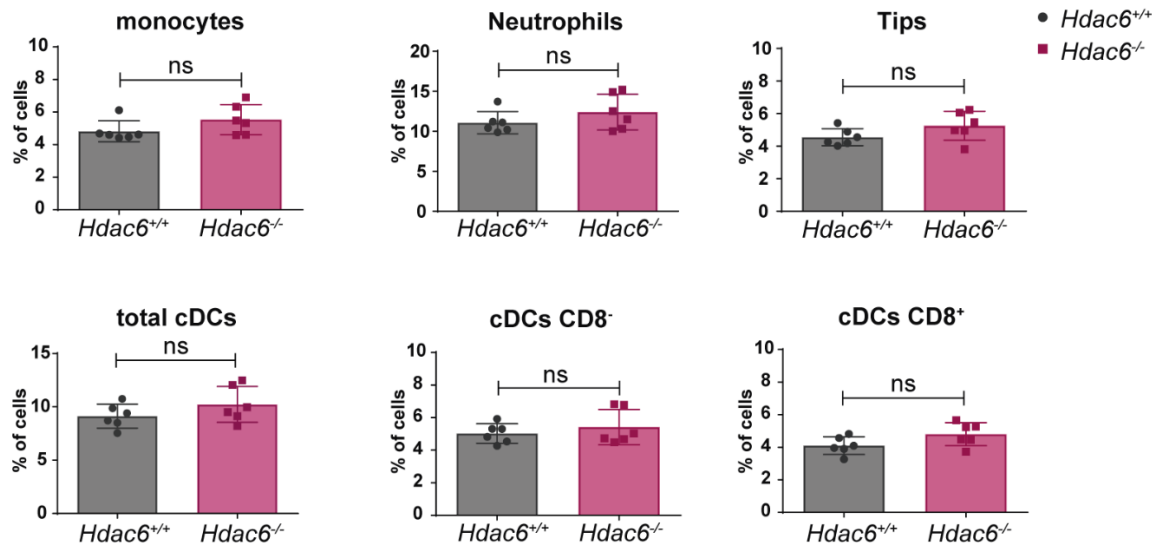
Next, to determine the specific cell populations underlying this phenotype, a multicolour gating strategy was used to identify the myeloid cell compartment, including monocytes, neutrophils, TIPS DCs, total cDCs, CD8<sup>-</sup> cDCs and CD8<sup>+</sup> cDCs (**Figure 4.14**).



**Figure 4.14. Gating strategy of myeloid populations of the spleen.** Dot-plots showing the gating of myeloid populations of spleen. Dot-plots showing SSC-A versus FSC-A indicates p1, FSC-H versus FSC-W and SSC-H versus SSC-W were used to avoid doublets and FSC-H versus viability shows live and dead cells. Singlets and live cells were used to choose CD3<sup>+</sup>CD19<sup>-</sup>DX5<sup>-</sup>Ly6G<sup>+</sup> cell population. From this population, neutrophils were gated as Ly6G<sup>+</sup>Ly6C<sup>+</sup> cells, monocytes as CD11b<sup>+</sup>CD11c<sup>lo</sup>, TIPS DCs as intermediate levels of CD11b and CD11c, conventional dendritic cells (cDCs) as CD11c<sup>hi</sup>; inside this population CD8<sup>-</sup> cDCs were distinguished as CD11c<sup>hi</sup>CD11b<sup>+</sup> and CD8<sup>+</sup> cDCs as CD11c<sup>hi</sup>CD11b<sup>lo</sup>.

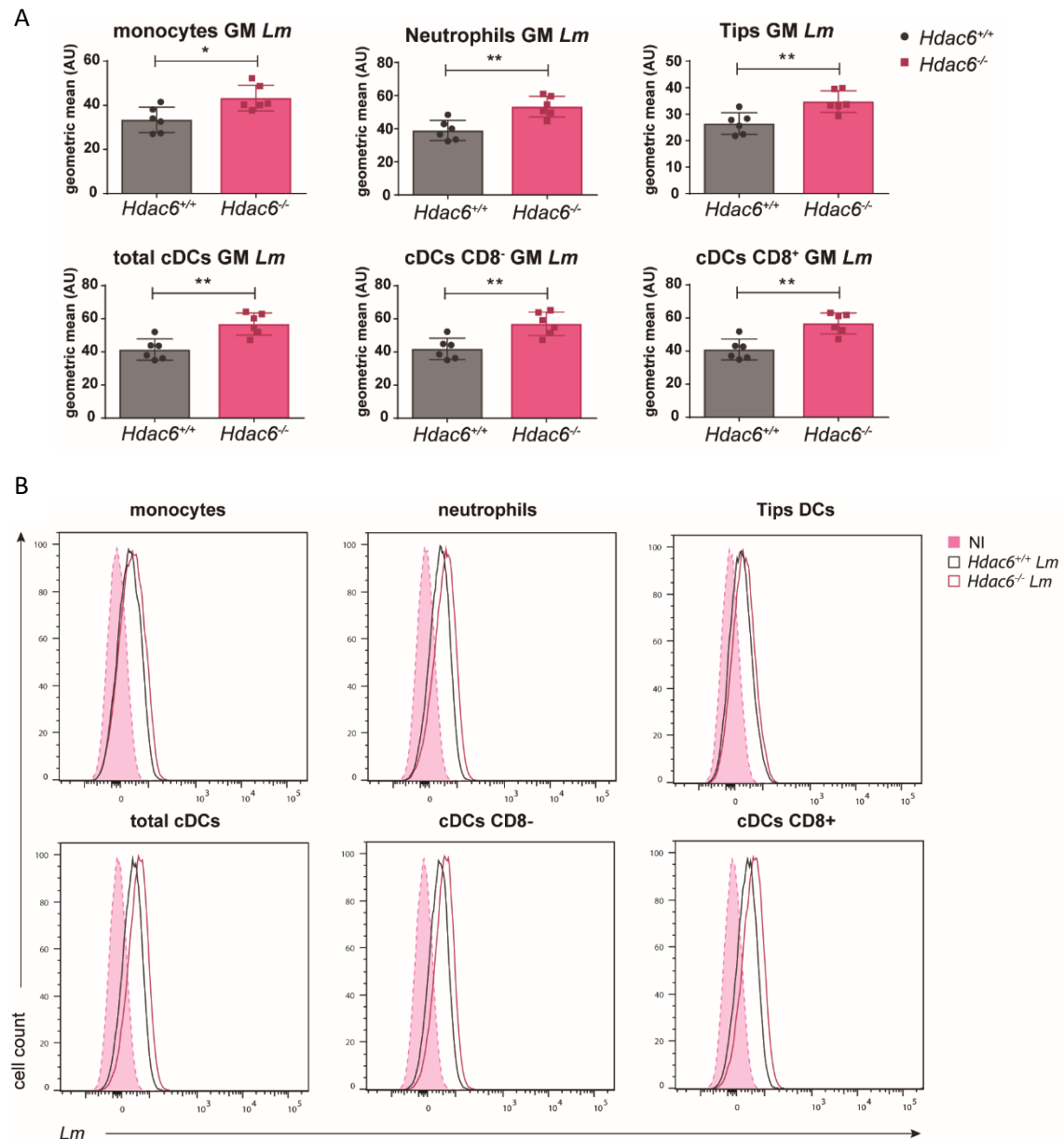


Non-significant differences were detected in the total number of myeloid splenic populations between *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> genotypes at 6 hpi of *Lm*-infection (**Figure 4.15**).



**Figure 4.15. Comparison of myeloid populations of the spleen between *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> genotypes.** The charts show percentage of cells of the different splenic populations (monocytes, neutrophils, Tregs DCs, total cDCs, CD8<sup>-</sup> cDCs and CD8<sup>+</sup> cDCs) gated in the live CD3<sup>+</sup>CD19<sup>-</sup>DX5<sup>-</sup> population of *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> mice injected with a lethal dose of *Lm* at 6 hpi. Two-tailed t-test, ns>0.05 non-significant; n=6.

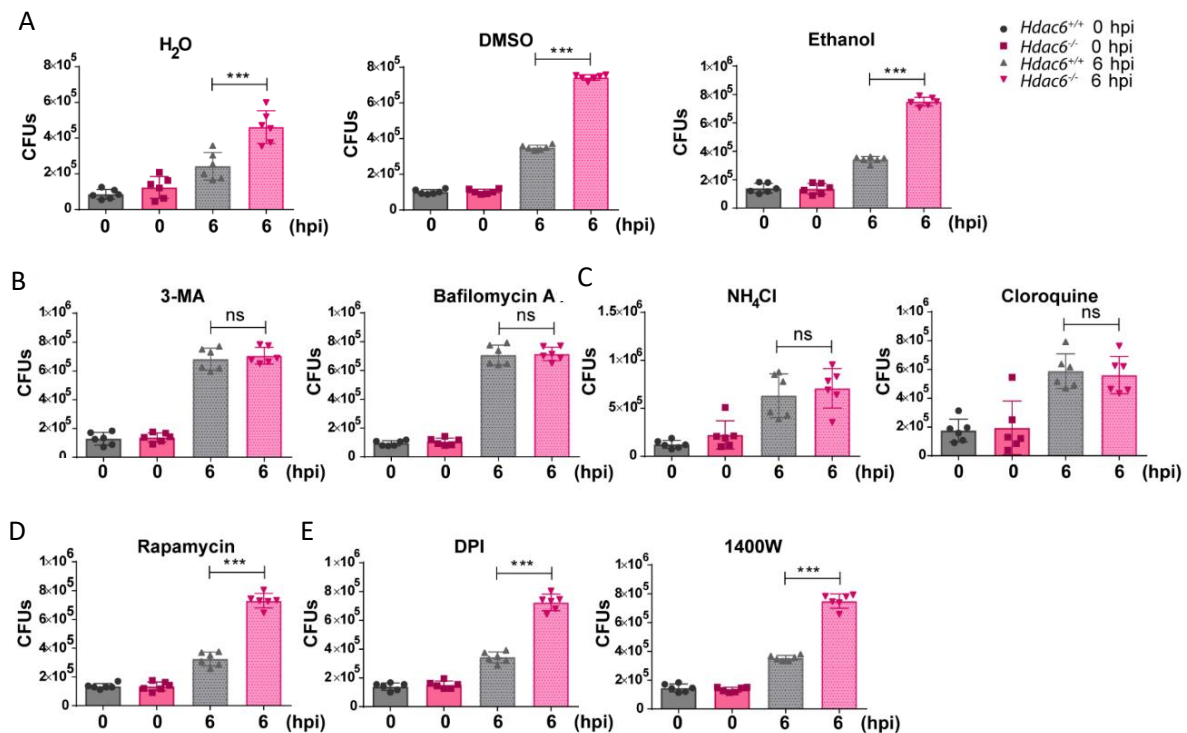
However, higher numbers of *Lm* were observed in different myeloid cells at 6 hpi (**Figure 4.16**). These data highlight the impairment of intracellular *Lm* clearance in *Hdac6*<sup>-/-</sup> myeloid cells.



**Figure 4.16. Deficient intracellular bacteria clearance in *Hdac6*<sup>-/-</sup> splenic myeloid populations.** (A) The charts show geometric means (GM) of *Lm* of different splenic populations (monocytes, neutrophils, Tips DCs, total cDCs, cDCs CD8<sup>-</sup> and cDCs CD8<sup>+</sup>) gated in the live CD3<sup>+</sup>CD19<sup>-</sup>DX5<sup>-</sup> population of *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> mice injected with a lethal dose of *Lm* at 6 hpi. Two-tailed t-test, \*\**p*≤0.01, \**p*≤0.05; *n*=6. (B) Representative histograms of different splenic populations (monocytes, neutrophils, Tips DCs, total cDCs, CD8<sup>-</sup> cDCs and CD8<sup>+</sup> cDCs) show *Lm* signal of *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> mice injected with a lethal dose of *Lm* at 6 hpi. A pool of *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> non-infected spleens was used as a control sample without infection (NI).

#### 4.1.4. Evaluation of cellular clearance mechanisms of *Lm*

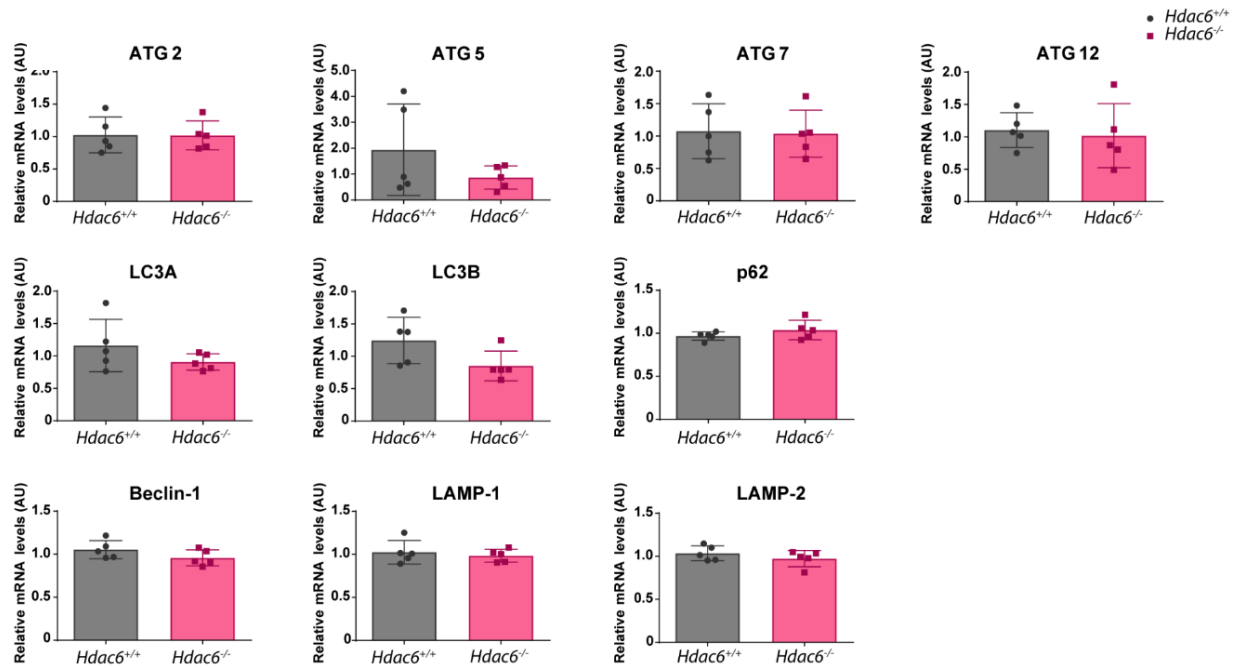
To test the involvement of autophagy in the mechanism by which HDAC6 regulates *Lm* infection, we treated GM-CSF-derived DCs with 3-methyladenine (3-MA), an inhibitor of autophagosome formation. Treatment with 3-MA increased bacterial load in *Hdac6*<sup>+/+</sup> DCs at 6 hpi, while having no effect on *Hdac6*<sup>-/-</sup> DCs (Figure 4.17. B), suggesting autophagy as the bacterial clearance mechanism impaired in *Hdac6*-deficient DCs. A similar result was observed upon treatment of DCs with bafilomycin A1, an inhibitor of vacuolar proton pump that indirectly inhibits phagosome-lysosome fusion, and with the lysosome acidification inhibitors chloroquine and NH<sub>4</sub>Cl (Figure 4.17. B and C). In contrast, increasing autophagy flux with rapamycin did not restore the impaired autophagy in *Hdac6*<sup>-/-</sup> DCs (Figure 4.17. D). No significant effects were observed with control vehicles (Figure 4.17. A). To explore other possible mechanisms, we treated DCs with inhibitors of NADPH oxidase (DPI) and iNOS (1400W). These treatments did not alter the difference in CFU number at 6 hpi between treated and non-treated *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> DCs, indicating that the activity of either enzyme is not accounting for the existing phenotype (Figure 4.17. E).



**Figure 4.17. Intracellular bacteria after treatment with different inhibitors of principal clearance mechanisms of the cell.** (A) Total CFUs at 0 and 6 hpi in *Lm*-infected GM-CSF DCs (MOI of 10) treated with different control vehicles (H<sub>2</sub>O, DMSO and ethanol). Total CFUs in *Lm*-infected DCs treated with inhibitors. CFUs were detected at entry (0 hpi) and 6 hpi (bacterial proliferation) using: (B) the autophagy inhibitors (3-MA and bafilomycin A1, (C) the lysosome acidification inhibitors (NH<sub>4</sub>Cl and chloroquine), and (D) the autophagy activator (rapamycin), (E) the NADPH oxidase inhibitor (DPI) and the iNOS inhibitor (1400W). Two-tailed t-test, \*\*\*p<0.001, ns>0.05 non-significant; n=6. Specification of control vehicles: H<sub>2</sub>O were the control vehicle used for NH<sub>4</sub>Cl and chloroquine, DMSO for 3-MA, bafilomycin A1, DPI and 1400W and ethanol for rapamycin. Time 0 hpi is included as a bacterial entry control.

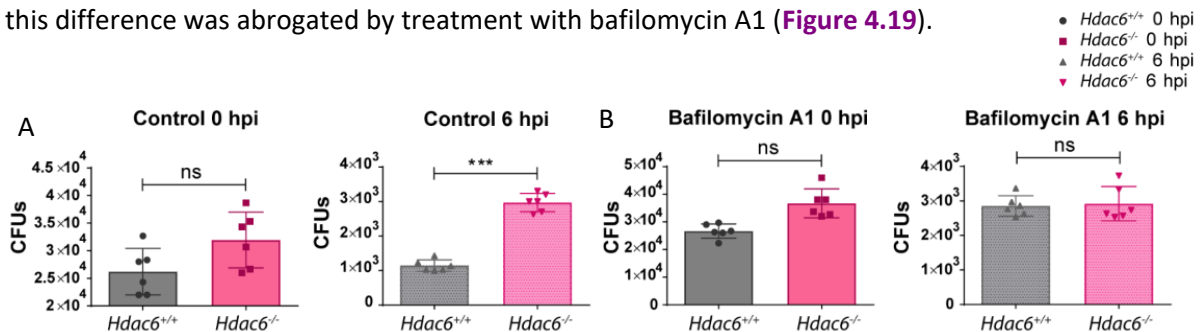
#### 4.1.5. Consequence of the lack of HDAC6 in the autophagy of *Lm*

The defective autophagy phenotype of *Hdac6*<sup>-/-</sup> GM-CSF-derived DCs was not due to transcriptional alterations of autophagy or lysosome components, since *Lm*-infected *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> DCs showed no mRNA expression differences at 6 hpi in the autophagy components LC3 A and B, p62, ATG2, 5, 7 and 12, and Beclin-1 or in the lysosome components LAMP-1 and 2 (Figure 4.18).



**Figure 4.18. Expression of relevant autophagy and lysosome markers in *Lm*-infected GM-CSF-DCs at 6 hpi.** PCR analysis of autophagy markers (ATG-2, 5, 7 and 12, LC3A and B, p62 and Beclin-1) and lysosome markers (LAMP-1 and 2) (AU: arbitrary units) after 6 hpi with *Lm*. Two-tailed t-test, ns>0.05 non-significant; n=5.

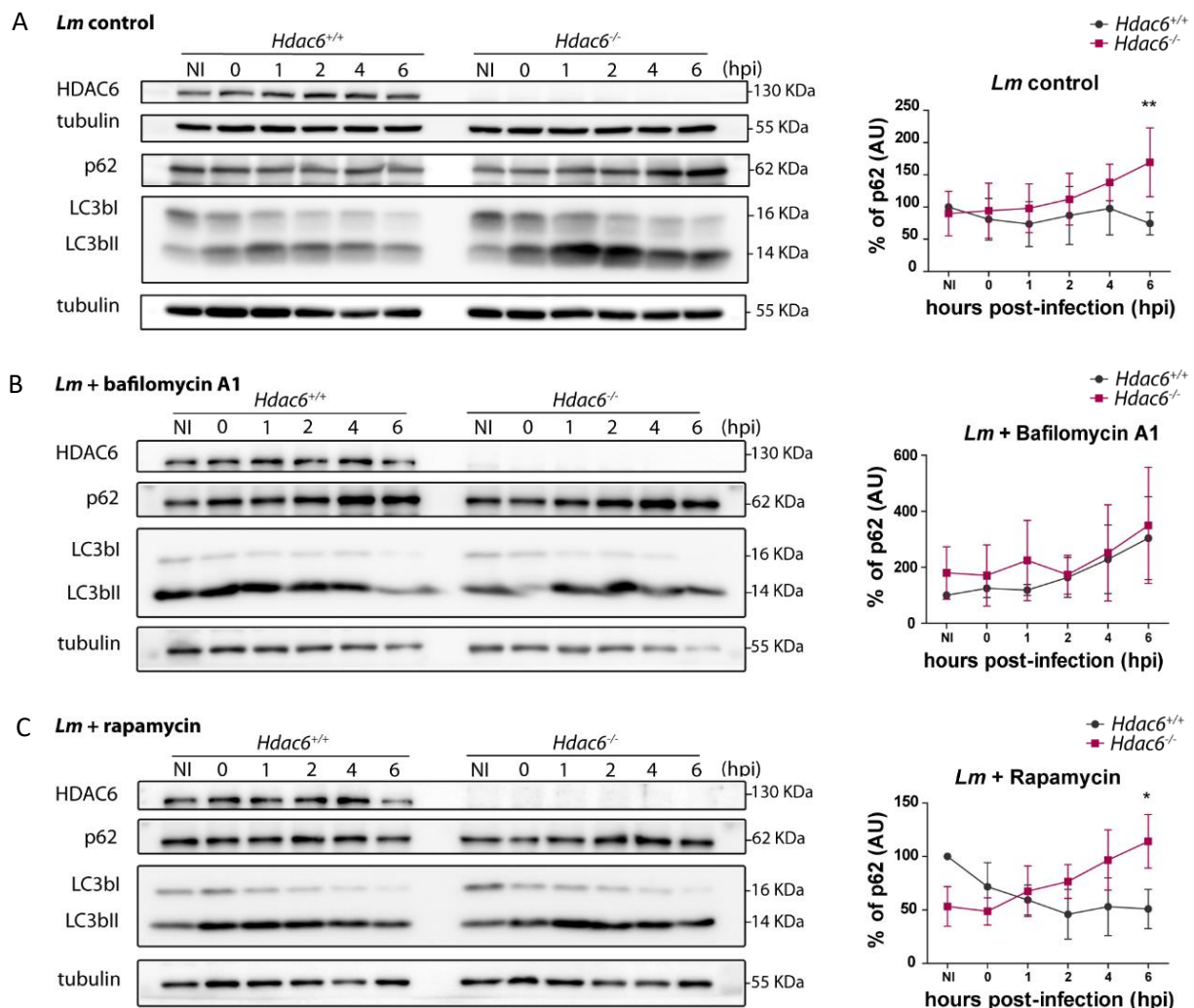
To determine whether these findings can be extended to other phagocytic cells, we carried out CFU assays with macrophages obtained from *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> mice four days after intraperitoneal thioglycollate injection. Higher bacterial load was observed only in *Hdac6*<sup>-/-</sup> macrophages at 6 hpi, and this difference was abrogated by treatment with bafilomycin A1 (Figure 4.19).



**Figure 4.19. Intracellular bacteria in peritoneal macrophages. (A)** Total CFUs at 0 and 6 hpi in *Lm*-infected thioglycollate-elicited macrophages. **(B)** Total CFUs at 0 and 6 hpi in *Lm*-infected TEMs treated with bafilomycin A1. Two-tailed t-test, \*\*\*p<0.001, ns>0.05 non-significant; n=6.

These data indicate that the phenotype observed in DCs is also extendable to other *Hdac6*-deficient phagocytic cells such as peritoneal macrophages, indicating a widespread defect in intracellular killing ability due to lack of HDAC6. Moreover, the killing ability shown by peritoneal macrophages is similar to that of M-CSF-derived macrophages and higher than GM-CSF-derived DCs (**Figure 4.19** compared with **Figure 4.10**).

To gain further insight into the autophagy mechanism affected by HDAC6, we monitored the autophagosome markers p62 and LC3bI and II in *Lm*-infected GM-CSF DCs (**Figure 4.20**).

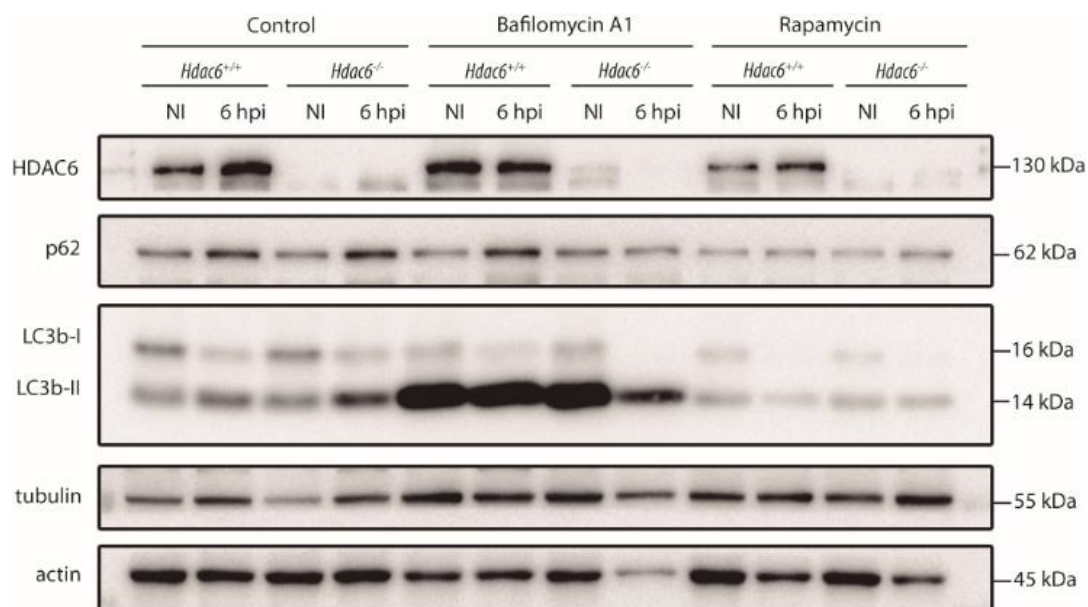


**Figure 4.20. Western-blot levels of autophagy markers during infection with *Lm* in GM-CSF DCs treated with or without various autophagy inhibitors or an autophagy inducer.** Left panels: Western-blot analysis of autophagy markers over the time-course of *Lm* infection in *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> DCs. Left panels: Levels of p62, LC3bI and II and HDAC6 were detected in (A) control cells and cells treated with (B) the inhibitor of phagosome maturation bafilomycin A1 and (C) the activator of autophagy rapamycin. Tubulin was used as a loading control. HDAC6 was used as a genotype check of *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> DCs and to monitor HDAC6 induction during infection. Right panels: Accompanying charts show quantification of the p62 percentage of control, bafilomycin A1 and rapamycin western blots in non-infected cells (NI) and 0, 1, 2, 4 and 6 hpi. NI time was considered the 100% point to refer other times as percentage from this sample. Two-tailed t-test, \*\*  $p \leq 0.01$ , \*  $p \leq 0.05$ , ns  $> 0.05$  non-significant; n=5.

*Hdac6*<sup>-/-</sup> DCs showed a 2-fold higher accumulation of p62 than *Hdac6*<sup>+/+</sup> cells at 6 hpi and increased LC3bII level in *Hdac6*<sup>+/+</sup> cells from 1 to 6 hpi (Figure 4.20. A). However, differences in p62 and LC3b levels were not found at early times of *Lm* infection of *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> DCs, indicating that the induction of autophagy is not affected in the absence of HDAC6 (Figure 4.20. A).

The treatment with bafilomycin A1 enhances the accumulation of p62 during the infection up to the same level in both genotypes, abrogating the deficiency in autophagy observed in *Hdac6*<sup>+/+</sup> DCs (Figure 4.20. B). Although rapamycin also increased p62 accumulation at early times in *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> DCs, only *Hdac6*<sup>+/+</sup> cells are able to diminish p62 at 6 hpi (Figure 4.20. C). This treatment confirmed the results obtained in the CFUs functional assays with this inhibitor (Figure 4.20. C compared with Figure 4.17. B). The similarity of the autophagy defect detected in *Hdac6*<sup>-/-</sup> DCs in control condition to that in rapamycin-treated *Hdac6*<sup>-/-</sup> cells, suggests an impairment in phagocytic vesicle fusion with the lysosome.

To show all the information about autophagy drugs in a single Western blot, samples of non-infected *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> DCs and *Lm*-infected at 6 hpi, without inhibitors, and treated with an autophagy inhibitor (bafilomycin A1) and an activator of autophagy (rapamycin) were shown (Figure 4.21). In accordance with data of the previous figure, an increment in p62 level in control *Hdac6*<sup>-/-</sup> DCs can be observed at 6 hpi, similar p62 levels in bafilomycin A1-treated *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> DCs at 6 hpi, and more p62 signal in rapamycin-treated *Hdac6*<sup>-/-</sup> DCs (Figure 4.21).

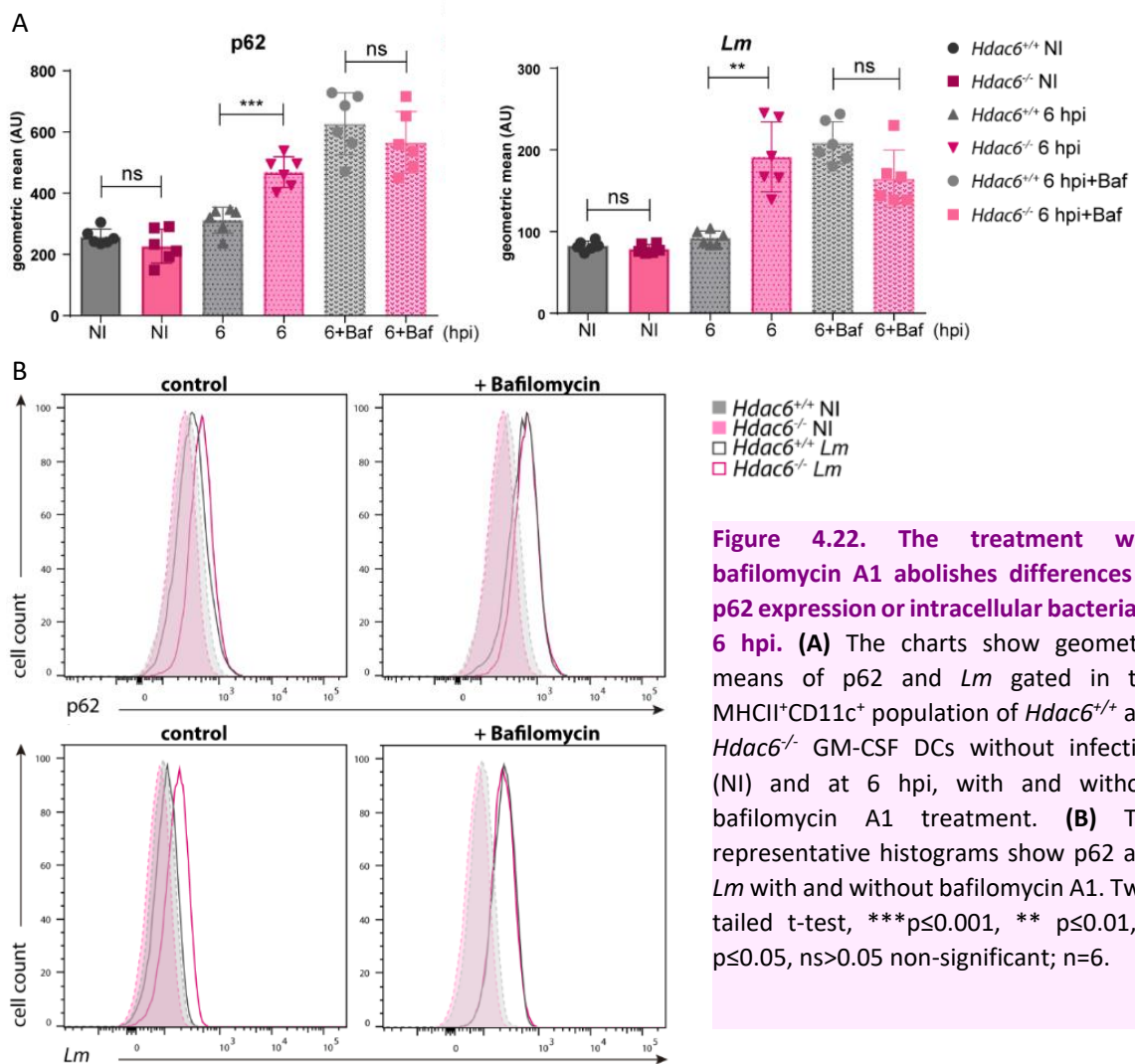


**Figure 4.21. Western-blot levels of autophagy markers in untreated and treated with bafilomycin A1 and rapamycin DCs.** Western-blot analysis of autophagy markers during *Lm* infection in *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> GM-CSF-derived DCs without treatment and treated with bafilomycin A1 and rapamycin. Levels of p62, LC3bI and II and HDAC6 were detected. Tubulin and actin were used as a loading controls. HDAC6 was as a genotype check of *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> DCs and to monitor HDAC6 induction during infection.



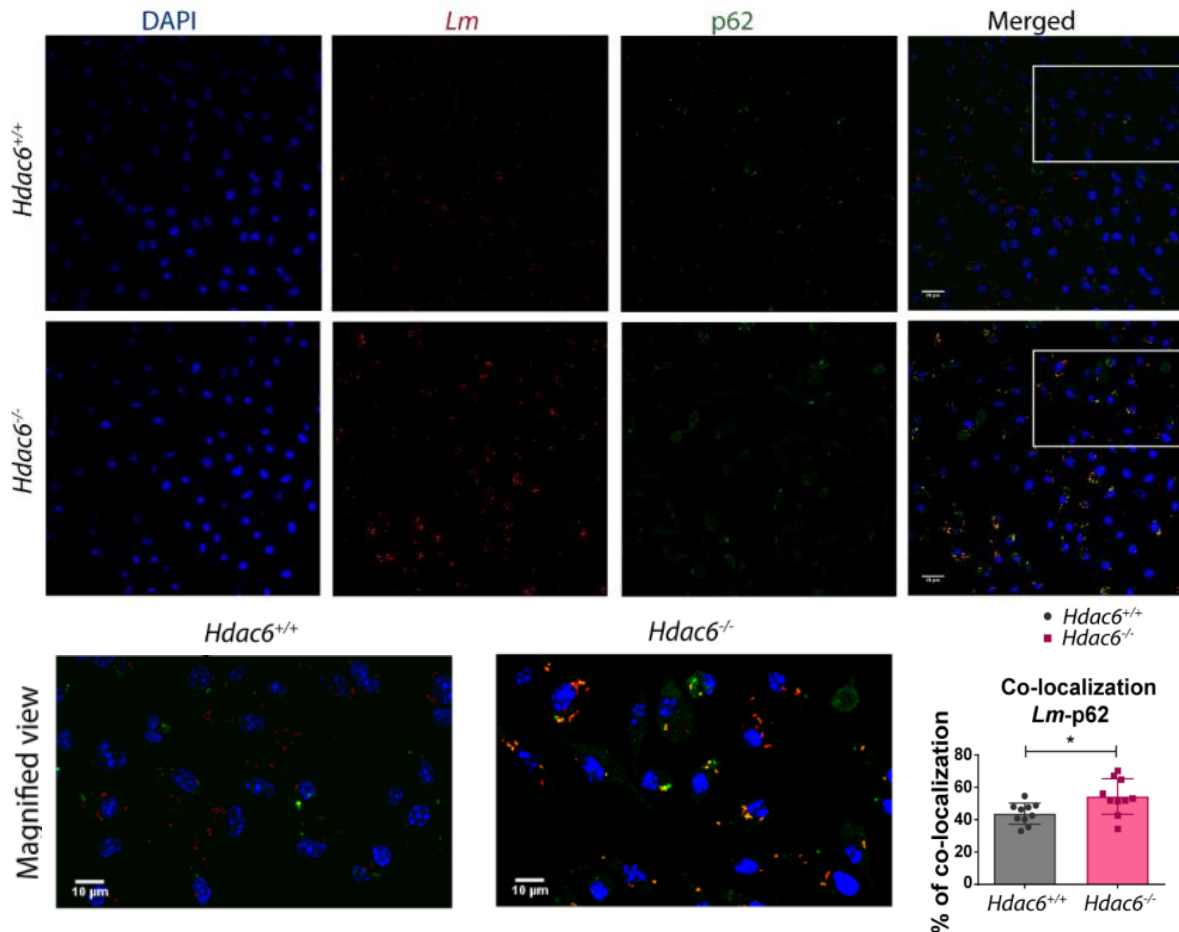
#### 4.1.6. Effect of HDAC6 absence in the accumulation of autophagy marker p62

In order to further understand the defective autophagy of *Hdac6*<sup>-/-</sup> DCs, the accumulation of p62 was studied in more detail. Flow cytometry at 6 hpi revealed significantly higher p62 content in *Hdac6*<sup>-/-</sup> GM-CSF DCs, indicating accumulation of this phagosome marker due to defective fusion of this organelle with the lysosome (Figure 4.22). Bafilomycin A1 treatment completely abrogated this difference, suggesting that *Hdac6*<sup>-/-</sup> DCs displayed an impairment in the final step of autophagy (Figure 4.22). More signal of *Lm* is displayed in *Hdac6*<sup>-/-</sup> DCs (Figure 4.22). In this regard, bafilomycin A1 treatment increased the low *Lm* signal in *Hdac6*<sup>+/+</sup> DCs to the level observed in *Hdac6*<sup>-/-</sup> cells (Figure 4.22).



**Figure 4.22.** The treatment with bafilomycin A1 abolishes differences in p62 expression or intracellular bacteria at 6 hpi. **(A)** The charts show geometric means of p62 and *Lm* gated in the MHCII<sup>+</sup>CD11c<sup>+</sup> population of *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> GM-CSF DCs without infection (NI) and at 6 hpi, with and without bafilomycin A1 treatment. **(B)** The representative histograms show p62 and *Lm* with and without bafilomycin A1. Two-tailed t-test, \*\*\*p≤0.001, \*\* p≤0.01, \* p≤0.05, ns>0.05 non-significant; n=6.

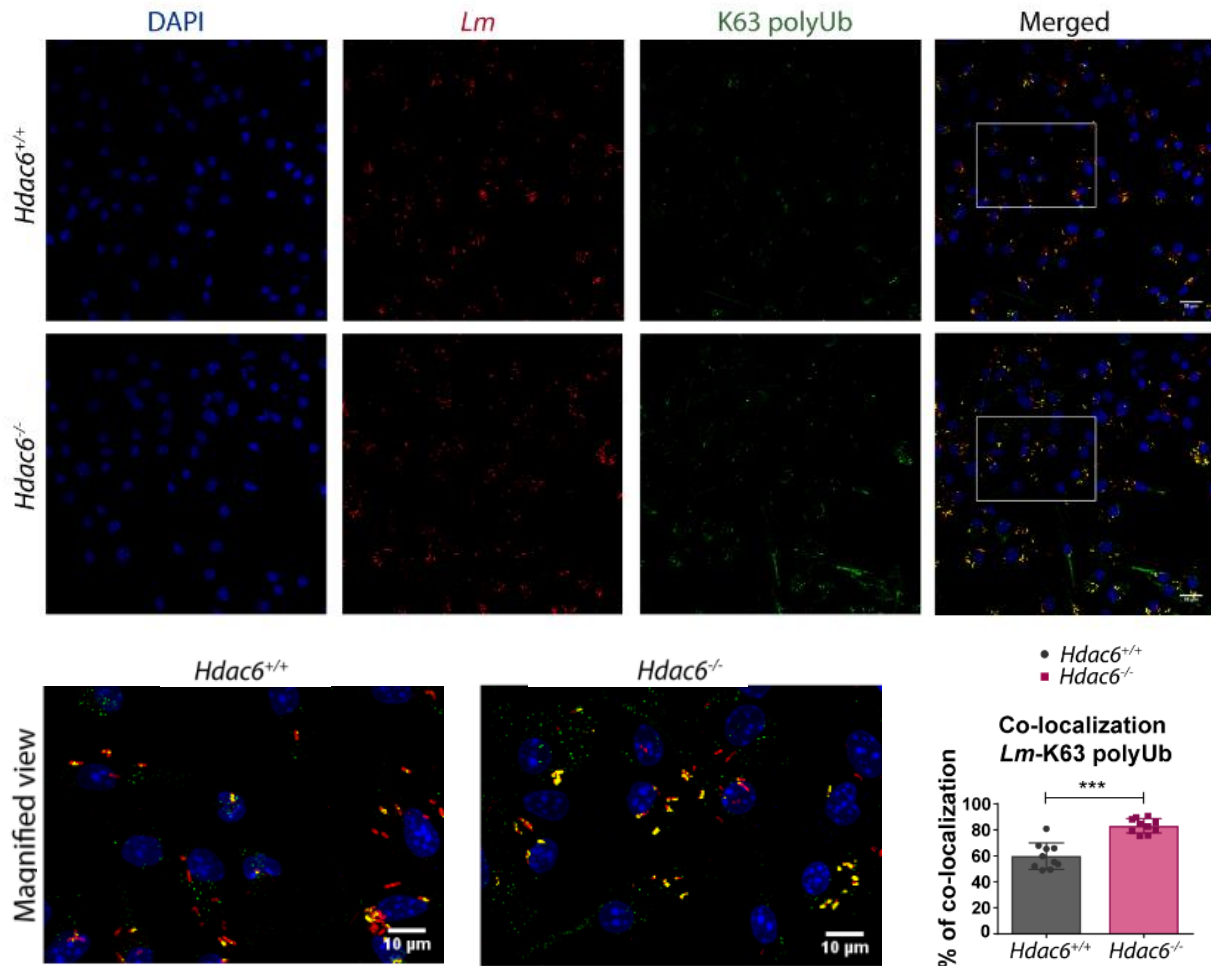
Confocal fluorescence analysis of *Lm*-infected DCs revealed increased levels of p62 in *Hdac6*<sup>-/-</sup> GM-CSF DCs (Figure 4.23). *Hdac6*<sup>-/-</sup> DCs also showed a higher percentage of p62-*Lm* co-localization than *Hdac6*<sup>+/+</sup> cells, indicating that *Hdac6*<sup>-/-</sup> cells have higher number of phagosome-contained bacteria (Figure 4.23 in accordance with p62 accumulation observed in Figure 4.20. A and Figure 4.22).



**Figure 4.23. Confocal microscopy of the phagosome marker p62 co-localization with *Lm*.** Confocal microscopy analysis of p62-*Lm* co-localization in *Lm*-infected *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> DCs at 6 hpi. *Left panel:* Panels show DAPI (blue), *Lm* (red), p62 (green), and merged views of the three channels, with magnified views of the boxed areas. Yellow indicates p62-*Lm* co-localization. Scale bars 20  $\mu$ m (main panels) and 10  $\mu$ m (magnified views). *Right panel:* The chart shows ImarisCell Module analysis of the number of cells and the number of bacteria per cell in all pictures (10 pictures per genotype). Co-localization percentages were obtained by measuring the p62 channel on the bacterial surface using a threshold of 100. The statistical analysis of Imaris quantifications corresponds to the percentage of p62-*Lm* co-localization at 6 hpi. Two-tailed t-test, \*p<0.05; n=10.

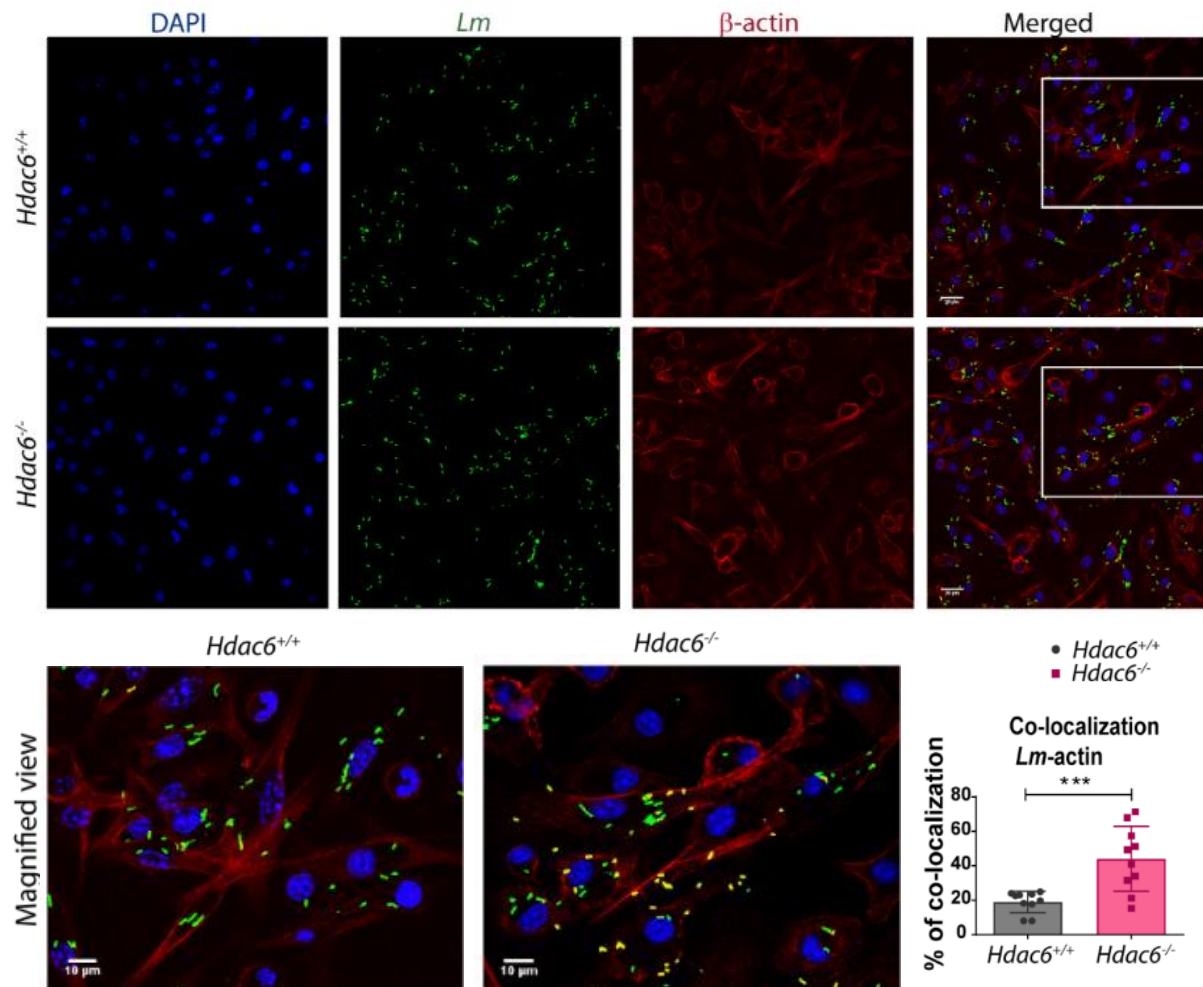


Increase of the autophagosomal marker p62 and the consequent accumulation of post-translational modification K63-linked poly-ubiquitin chains have been reported during impaired phagosome-lysosome fusion. Confocal fluorescence microscopy images of K63-linked poly-ubiquitin chains with *Lm* at 6 hpi reveal a higher accumulation of K63 poly-Ub in *Hdac6*<sup>-/-</sup> DCs, consistent with a stronger co-localization of bacteria tagged with this post-translational modification (PTM) (Figure 4.24).



**Figure 4.24. Confocal microscopy of K63 poly-ubiquitination co-localization with *Lm*.** Maximum intensity z-projections of confocal microscopy images of K63 polyUb-*Lm* co-localization in *Lm*-infected *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> DCs at 6 hpi. *Left panel:* Panels show DAPI (blue), *Lm* (red), K63 polyUb (green), and merged views of the three channels, with magnified views of the boxed areas. Yellow indicates *Lm*-K63 polyUb co-localization. Scale bars 20  $\mu$ m (main panels) and 10  $\mu$ m (magnified views). *Right panel:* The chart shows ImarisCell Module analysis of the number of cells and the number of bacteria per cell in all pictures (10 pictures per genotype). Co-localization percentages were obtained by measuring the K63 polyUb channel on the bacterial surface using a threshold of 82. The statistical analysis of Imaris quantifications corresponds to the percentage of K63 polyUb-*Lm* co-localization at 6 hpi. Two-tailed t-test, \*\*\*  $p \leq 0.001$ ;  $n=10$ .

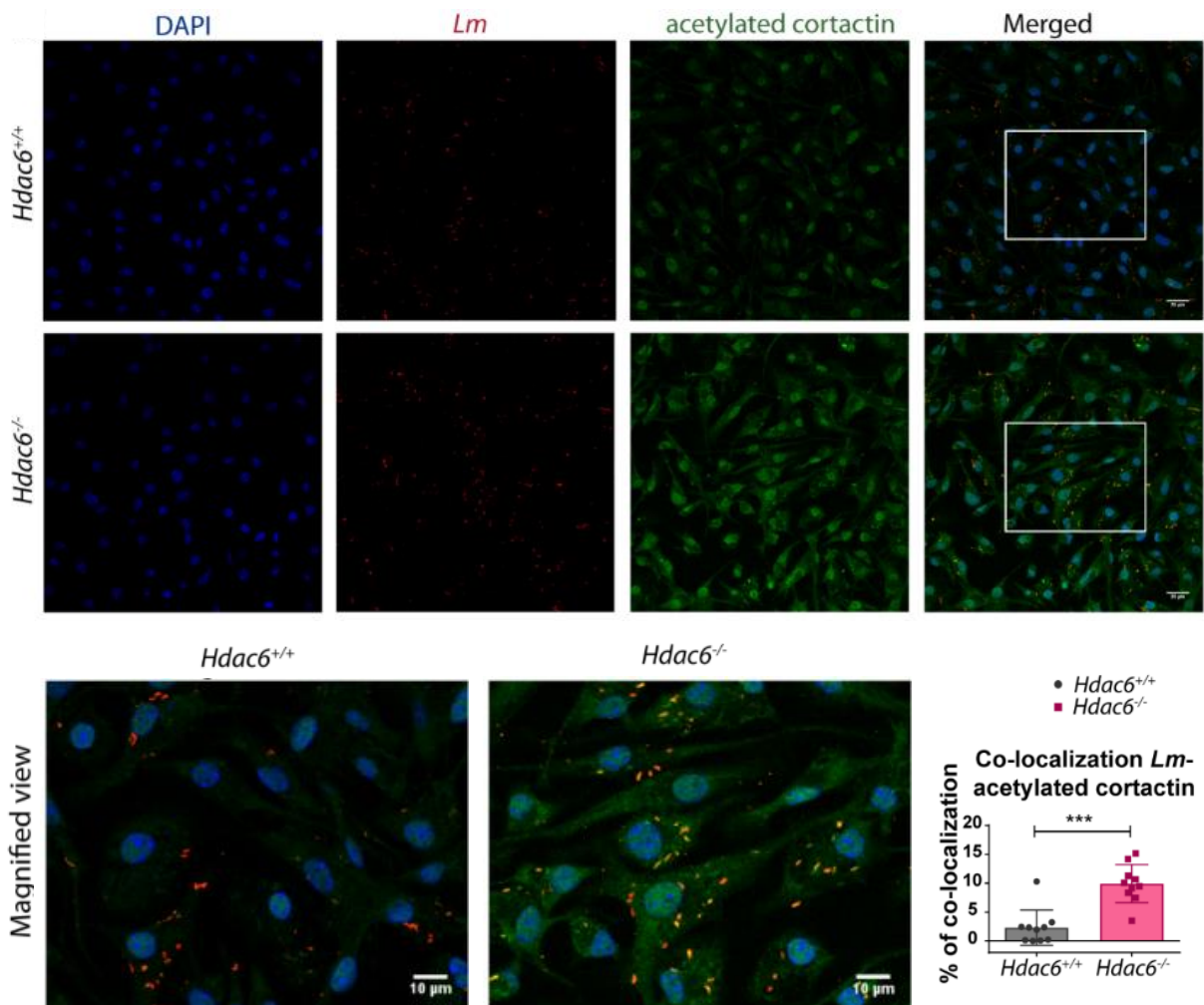
Confocal fluorescence microscopy study of actin and *Lm* revealed more frequent co-localization in *Hdac6*<sup>-/-</sup> than in *Hdac6*<sup>+/+</sup> DCs, indicating that more bacteria are at the cytoplasm to form actin rockets in *Hdac6*-deficient cells (**Figure 4.25**).



**Figure 4.25. Confocal microscopy of  $\beta$ -actin co-localization with *Lm*.** Confocal microscopy analysis of actin-*Lm* co-localization in *Lm*-infected *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> DCs at 6 hpi. **Left panel:** Panels show **DAPI (blue)**, ***Lm* (green)**,  **$\beta$ -actin (red)**, and merged views of the three channels, with magnified views of the boxed areas. Yellow indicates *Lm*- $\beta$ -actin co-localization. Scale bars 20  $\mu$ m (main panels) and 10  $\mu$ m (magnified views). **Right panel:** The chart shows ImarisCell Module analysis of the number of cells and the number of bacteria per cell in all pictures (10 pictures per genotype). Co-localization percentages were obtained by measuring the actin channel on the bacterial surface using a threshold of 40.6. The statistical analysis of Imaris quantifications corresponds to the percentage of actin-*Lm* co-localization at 6 hpi. Two-tailed t-test, \*\*\*  $p \leq 0.001$ ;  $n=10$ .

#### 4.1.7. Mechanism by which HDAC6 regulates autophagy of *Lm*

More signal of acetylated-cortactin is detected in *Hdac6*<sup>-/-</sup> DCs and also higher percentage of acetylated-cortactin-*Lm* co-localization (**Figure 4.26**).



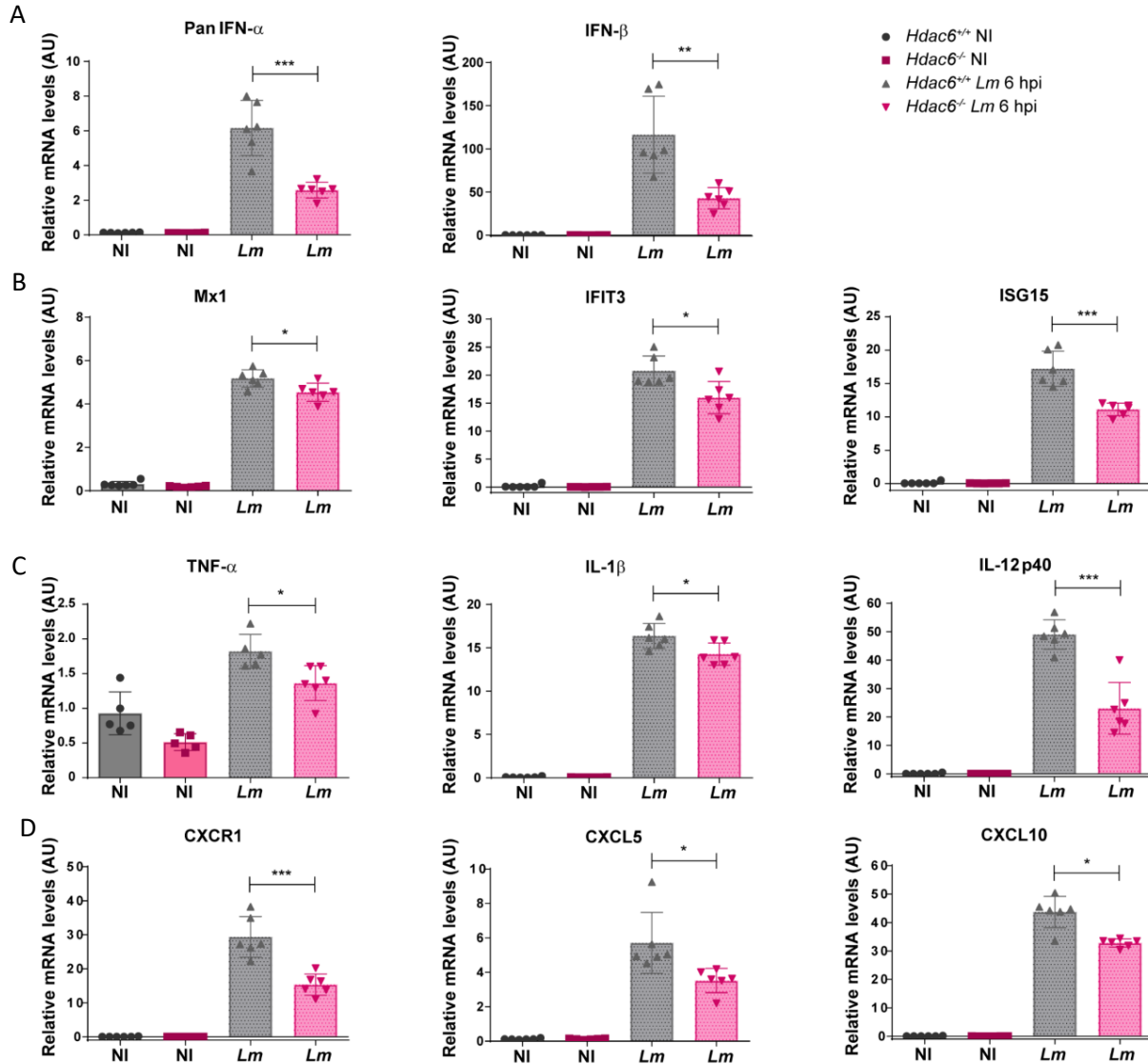
**Figure 4.26. Confocal microscopy of acetylated cortactin co-localization with *Lm*.** Confocal microscopy analysis of acetylated cortactin-*Lm* co-localization in *Lm*-infected *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> DCs at 6 hpi. *Left panel*: Panels show DAPI (blue), *Lm* (red), acetylated cortactin (green), and merged views of the three channels, with magnified views of the boxed areas. Yellow indicates *Lm*-acetylated cortactin co-localization. Scale bars 20 µm (main panels) and 10 µm (magnified views). *Right panel*: The chart shows ImarisCell Module analysis of the number of cells and the number of bacteria per cell in all pictures (10 pictures per genotype). Co-localization percentages were obtained by measuring the acetylated cortactin channel on the bacterial surface using a threshold of 184. The statistical analysis of Imaris quantifications corresponds to the percentage of acetylated cortactin-*Lm* co-localization at 6 hpi. Two-tailed t-test, \*\*\*  $p \leq 0.001$ ;  $n=10$ .

These data could explain the accumulation of p62 and the delay in phagocytic vesicle fusion observed in *Hdac6*<sup>-/-</sup> DCs, necessary to degrade phagocytosed *Lm*.

## 4.2. Study of the role of HDAC6 in the activation of dendritic cell functions by bacterial infection and stimulation of TLRs

### 4.2.1. Determination of pro-inflammatory genes expression

The effect of HDAC6 on the response of DCs to *Lm* was evaluated by measuring pro-inflammatory cytokine gene induction. The relative mRNA levels of type I interferons (interferons  $\alpha$  and  $\beta$ ) were lower in *Hdac6*<sup>-/-</sup> DCs at 6 hpi (Figure 4.27. A).



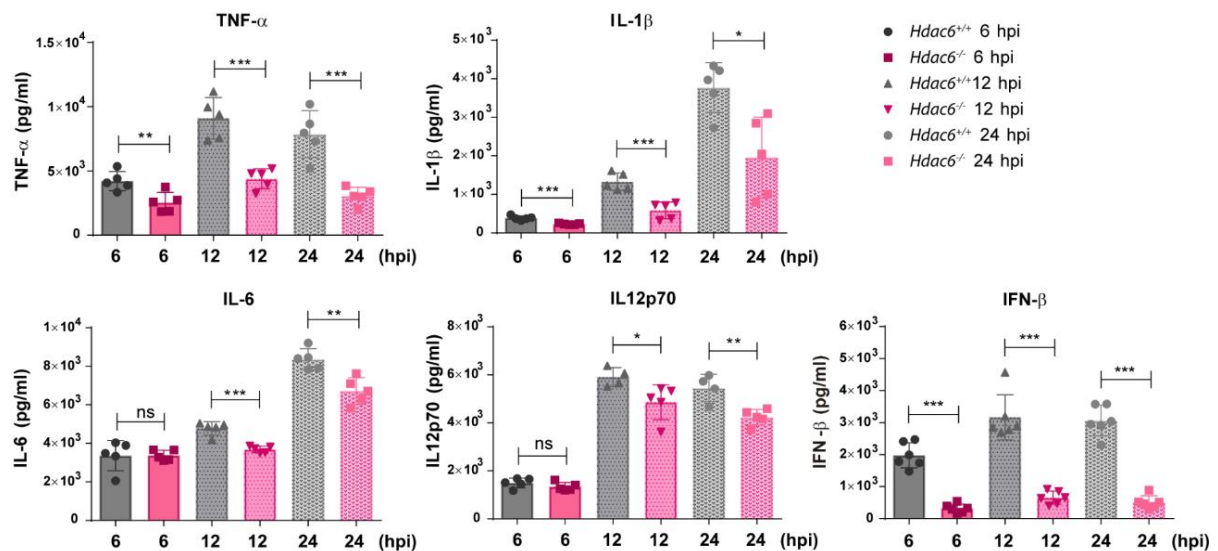
**Figure 4.27. Expression of Type I interferons, interferon down-stream proteins, pro-inflammatory cytokines, chemokine receptor and chemokines in DCs after *Lm* infection at 6 hpi.** PCR analysis of (A) Type-I interferons (PanIFN- $\alpha$  and IFN- $\beta$ ), (B) interferon downstream proteins (Mx1, IFIT3 and ISG15), (C) pro-inflammatory cytokines (TNF- $\alpha$ , IL-1 $\beta$  and IL-12p40), (D) chemokine receptor (CXCR1) and chemokines (CXCL5 and CXCL10) of *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> DCs non-infected (NI) and infected with *Lm* at 6 hpi (AU: arbitrary units). Two-tailed t-test, \*\*\* $p \leq 0.001$ , \*\* $p \leq 0.01$ , \* $p \leq 0.05$ ; n=5-6.



Accordingly, expression of downstream interferon-response genes such as Mx1, IFN-induced protein with tetratricopeptide repeats 3 (IFIT3), and ISG15 was also lower in *Hdac6*<sup>-/-</sup> DCs (Figure 4.27. B). Lack of HDAC6 also decreased the relative mRNA levels of the pro-inflammatory cytokines TNF $\alpha$ , IL-1 $\beta$  and IL12p40, indicating impaired cytokine activation after infection (Figure 4.27. C). Similarly, *Hdac6*<sup>-/-</sup> DCs expressed lower levels than their *Hdac6*<sup>+/+</sup> counterparts of the chemokine receptor CXCR1 and chemokines CXCL5 and CXCL10 (Figure 4.27. D). These data demonstrate that *Hdac6*-deficient DCs have a weakened activation response to *Lm* infection at 6 hpi, which suggests a defect in bacterial clearance, consistent with the increased bacterial load in these cells.

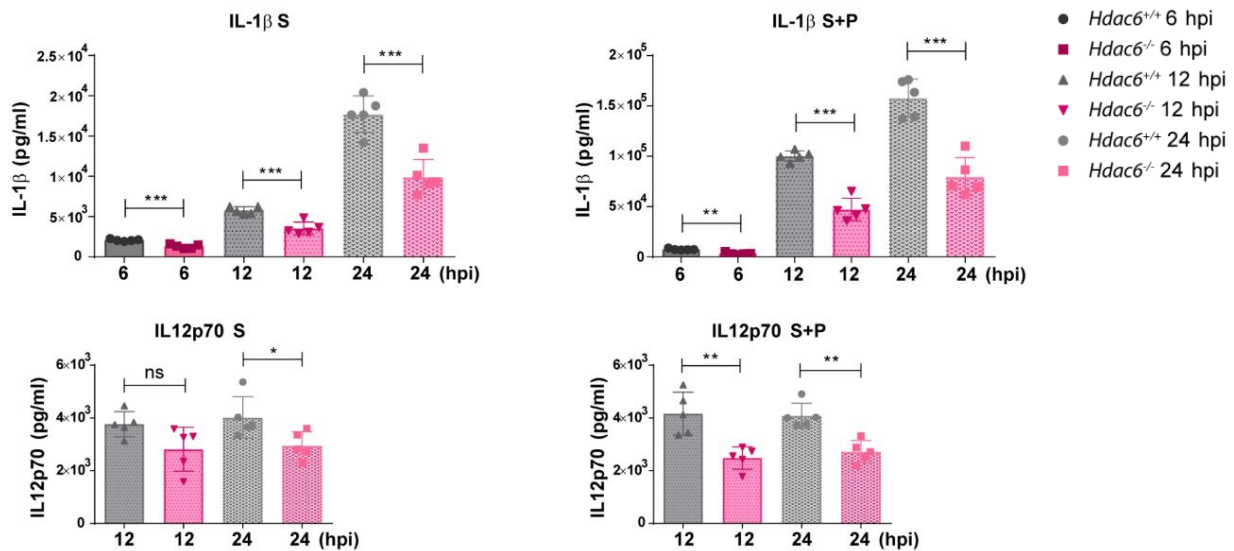
#### 4.2.2. Effect of HDAC6 in pro-inflammatory cytokine secretion after *Lm* infection

To confirm these data, we monitored pro-inflammatory cytokines and IFN- $\beta$  in the supernatants of *Lm*-infected DCs. Early after infection, TNF $\alpha$ , IL-1 $\beta$ , IL-6, IL12p70 and IFN- $\beta$  levels were lower in supernatants from *Hdac6*<sup>-/-</sup> cells than in those from *Hdac6*<sup>+/+</sup> cells, and this difference held at 12 and 24 hpi (Figure 4.28).



**Figure 4.28. Pro-inflammatory cytokines secreted by *Lm*-infected DCs.** ELISA analysis of the pro-inflammatory cytokines TNF $\alpha$ , IL1 $\beta$ , IL6 and IL12p70 (pg/ml) and IFN- $\beta$  in supernatants of *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> DCs at 6, 12 and 24 hpi with *Lm*. Two-tailed t-test, \*\*\*p<0.001, \*\* p<0.01, \* p<0.05 ns>0.05 non-significant; n=5-6.

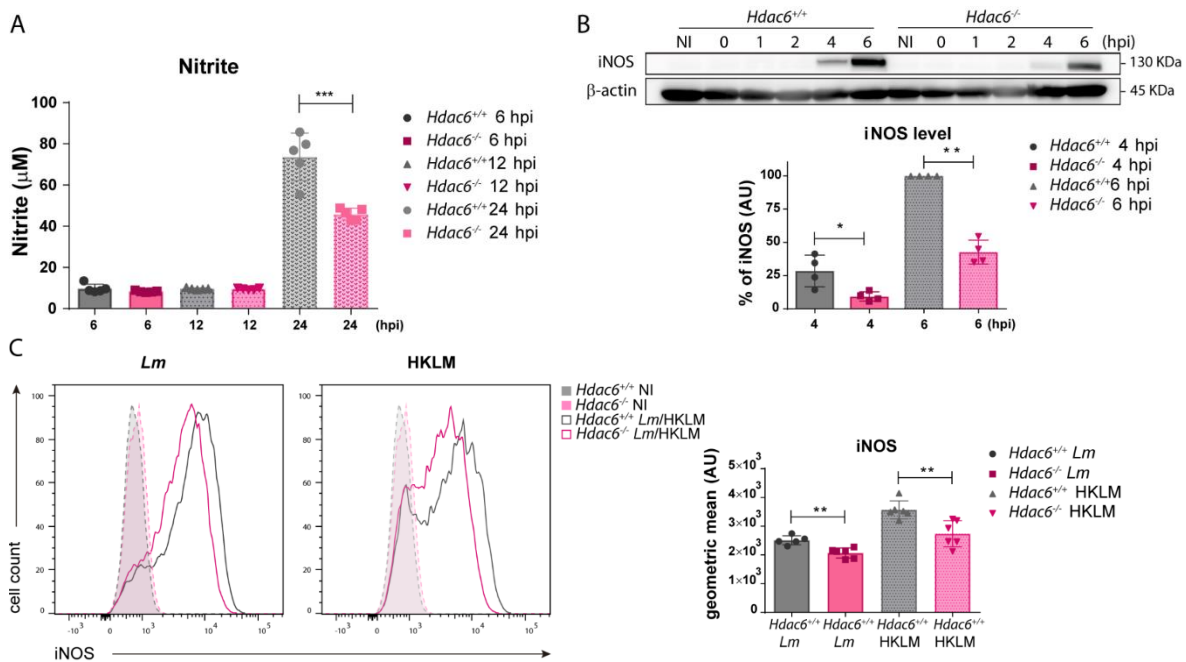
To exclude a defect in cytokine secretion, we compared cytokine levels in supernatants (S) with the levels in supernatants plus their corresponding cell pellets (S+P). Both analyses showed decreased cytokine levels in *Hdac6*<sup>-/-</sup> cells, indicating an impaired antibacterial response in *Hdac6*-deficient DCs (Figure 4.29).



**Figure 4.29. Comparison of secreted versus intracellular+secreted pro-inflammatory cytokines.** ELISA detection of the pro-inflammatory cytokines IL-1 $\beta$  and IL12p70 (pg/ml) in supernatants (S) and in supernatants plus the corresponding cell pellets (S+P) of *Lm*-infected *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> DCs at 6, 12 and 24 hpi. Two-tailed t-test, \*\*\* $p \leq 0.001$ , \*\* $p \leq 0.01$ , \* $p \leq 0.05$ , ns>0.05 non-significant; n=5.

#### 4.2.3. Influence of HDAC6 in the microbicidal activity of iNOS

Measurement of nitrite in supernatants of infected-DCs revealed higher nitric oxide production by *Hdac6*<sup>+/+</sup> DCs than in *Hdac6*<sup>-/-</sup> DCs at 24 hpi (Figure 4.30. A).

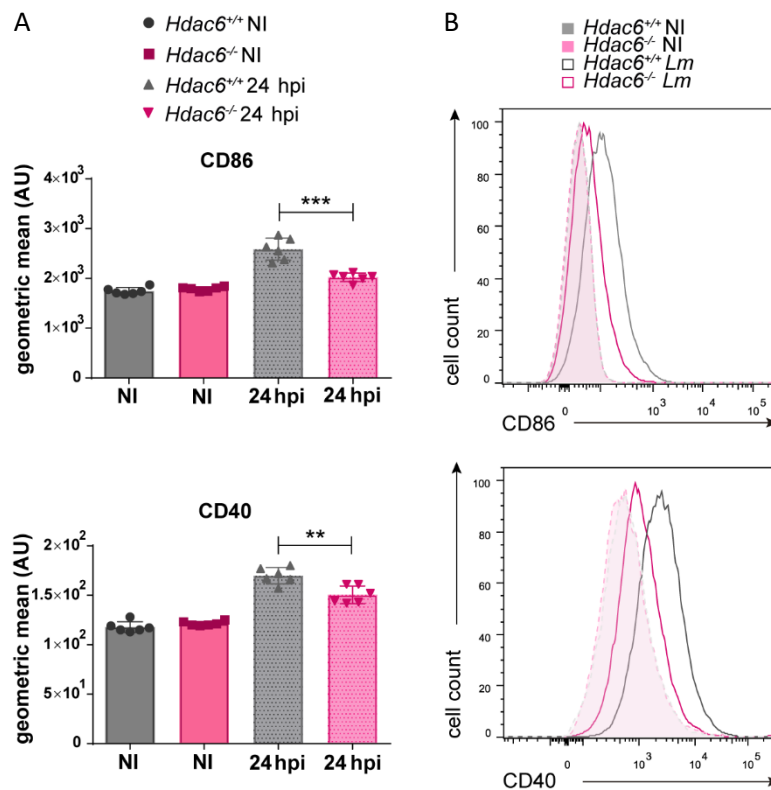


**Figure 4.30. *Lm* activated iNOS activity in DCs.** (A) Nitrite levels in supernatants of *Lm*-infected DCs at 6, 12 and 24 hpi. Two-tailed t-test, \*\*\* $p \leq 0.001$ ; n=5. (B) Top panel: Western-blot analysis of iNOS over the time-course of infection.  $\beta$ -actin was used as a loading control. Lower panel: The chart shows quantification of iNOS at 4 and 6 hpi. Two-tailed t-test, \*\* $p \leq 0.01$ , \* $p \leq 0.05$ ; n=4 (C) Left: The panel shows representative histograms of iNOS expressed by *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> DCs after exposure to live *Lm* or HKLM for 24 h (left). Right: The chart shows the geometric mean of iNOS expression. Non-infected (NI) DCs were used as a control of iNOS induction. Two-tailed t-test, \*\* $p \leq 0.01$ ; n=6.

In agreement, western blot revealed lower levels of inducible nitric oxide synthase (iNOS) in *Hdac6*<sup>-/-</sup> DCs at 4 and 6 hpi (Figure 4.30. B), indicating a delay of the enzyme induction in *Hdac6*<sup>-/-</sup> DCs. Likewise, flow cytometry after exposure of DCs to live or heat-killed *Listeria monocytogenes* (HKLM) revealed higher expression of iNOS in *Hdac6*<sup>+/-</sup> DCs in both cases (Figure 4.30. C). These data support the involvement of HDAC6 in the activation of DC-mediated iNOS microbicidal responses to *Lm* infection and in the clearance of this intracellular pathogen.

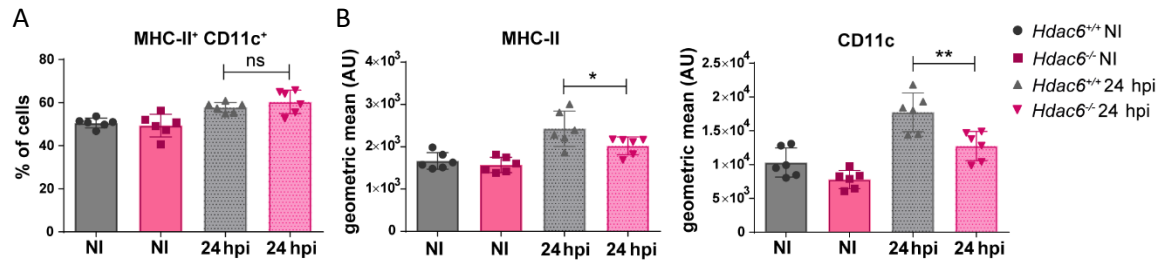
#### 4.2.4. Study of the expression of MHC co-stimulatory molecules on the membrane

Flow cytometry assay of *Lm* infected-GM-CSF-DCs at 24 hpi showed higher level of expression of membrane CD86 and CD40, both molecules routinely used as markers of co-stimulation of MHC (Figure 4.31).



**Figure 4.31. CD86 and CD40 membrane expression after *Lm* infection.** (A) The charts show geometric means of CD86 and CD40 gated in live MHCII<sup>+</sup>CD11c<sup>+</sup> population of *Hdac6*<sup>+/-</sup> and *Hdac6*<sup>-/-</sup> DCs without infection (NI) and 24 h after exposure to *Lm*. Two-tailed t-test, \*\*\*p<0.001, \*\* p<0.01, ns>0.05 non-significant; n=6. (B) The representative histograms show CD86 and CD40. Non-infected NI DCs were used as a control of CD86 and CD40 induction.

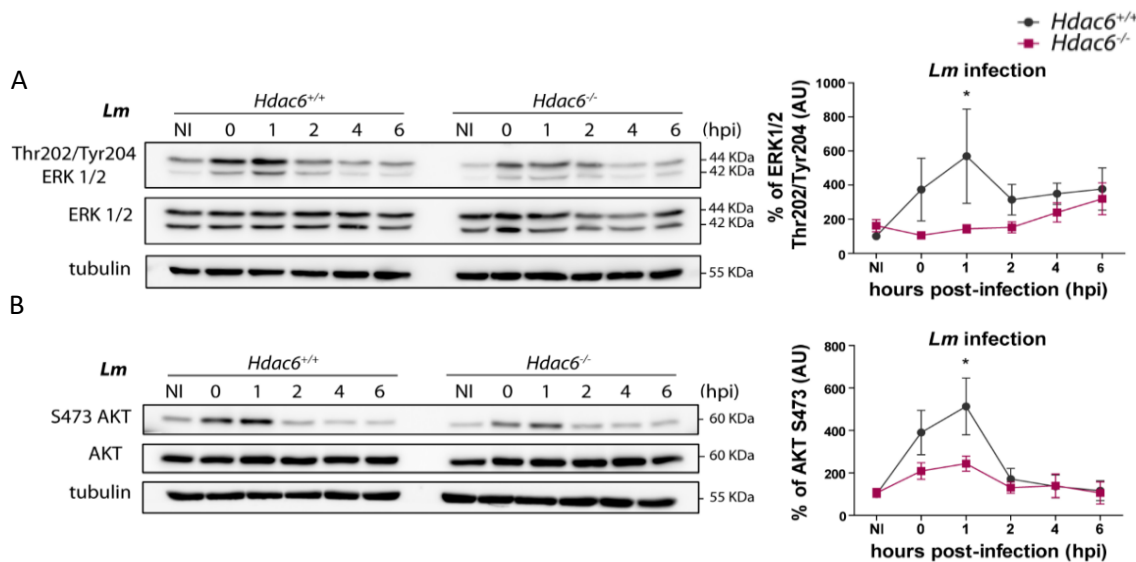
The MHC-II+CD11c<sup>+</sup> population of DCs were gated to check geometric means of CD86 and CD40, and non-significant differences in the percentage of this population were detected between *Hdac6*<sup>+/-</sup> and *Hdac6*<sup>-/-</sup> DCs (Figure 4.32). However, the MHC-II+CD11c<sup>+</sup> population express higher levels of MHC-II and CD11c molecules on the cell surface in *Hdac6*<sup>+/-</sup> DCs (Figure 4.32). These results are in accordance with the observation of iNOS and pro-inflammatory cytokines, supporting the hypothesis of a stronger activation of *Hdac6*<sup>+/-</sup> DCs after *Lm* infection than *Hdac6*<sup>-/-</sup> ones.



**Figure 4.32. MHC-II and CD11c membrane expression after activation of DCs with *Lm* infection.** (A) The chart displays the percentage of live cells of *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> DCs gated as MHC-II<sup>+</sup>CD11c<sup>+</sup>. Non-infected (NI) and 24 hpi *Lm*-infected conditions were tested. Two-tailed t-test, ns>0.05 non-significant; n=6. (B) The charts show geometric means of MHC-II and CD11c gated in live MHCII<sup>+</sup>CD11c<sup>+</sup> population of *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> DCs without infection (NI) and 24 h after infection with *Lm*. Two-tailed t-test, \*\* p≤0.01, \* p≤0.05, ns>0.05 non-significant; n=6.

#### 4.2.5. Function of HDAC6 in the activation of MAPK- and mTOR-signalling pathways

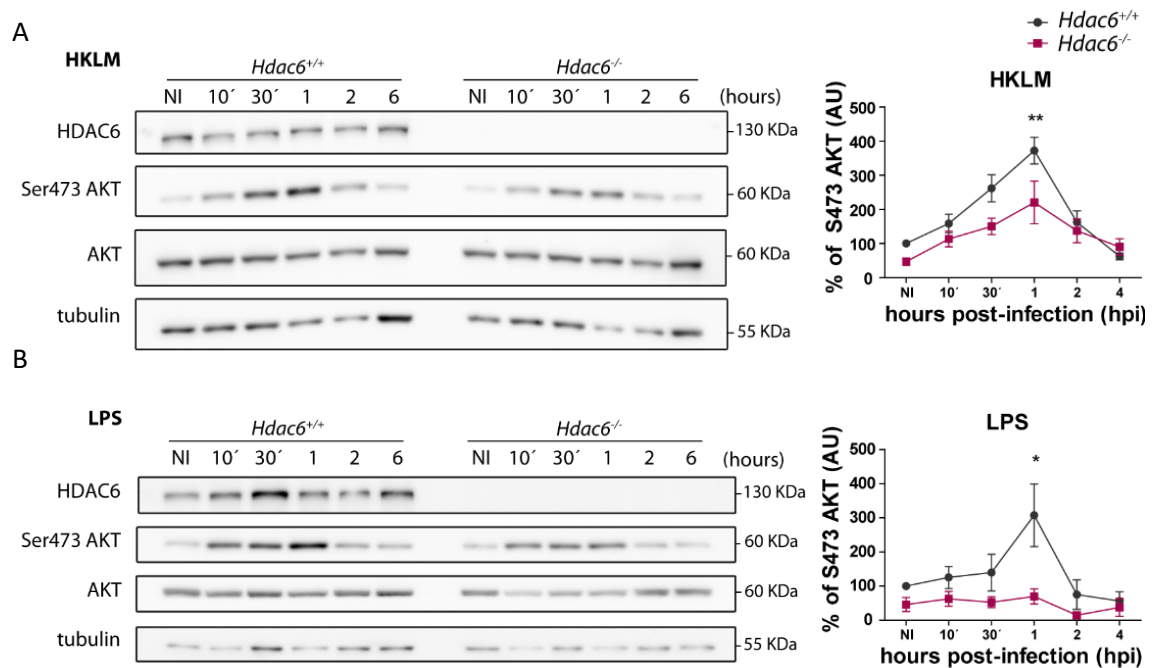
The diminished activation response against *Lm* in *Hdac6*<sup>-/-</sup> DCs is consistent with impaired TLR-related signalling. To investigate this question, we determined the phosphorylation levels of TLR downstream mediators by western blot. Compared with *Hdac6*<sup>+/+</sup> DCs, *Hdac6*<sup>-/-</sup> DCs showed weaker phosphorylation signals for ERK and AKT after *Lm* infection (Figure 4.33. A and B).



**Figure 4.33. MAPK and AKT activation after *Lm* infection.** Western-blot analysis of MAPK activation ERK1/2 and AKT over the time-course of *Lm* infection in *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> DCs. Left: Total and phosphorylated (A) ERK and (B) AKT were detected. Tubulin was used as a loading control. Right: Accompanying charts show quantification of (A) pERK/totalERK and (B) pAKT/totalAKT ratios relative to the loading control, ns non-significant; n=7 (right).

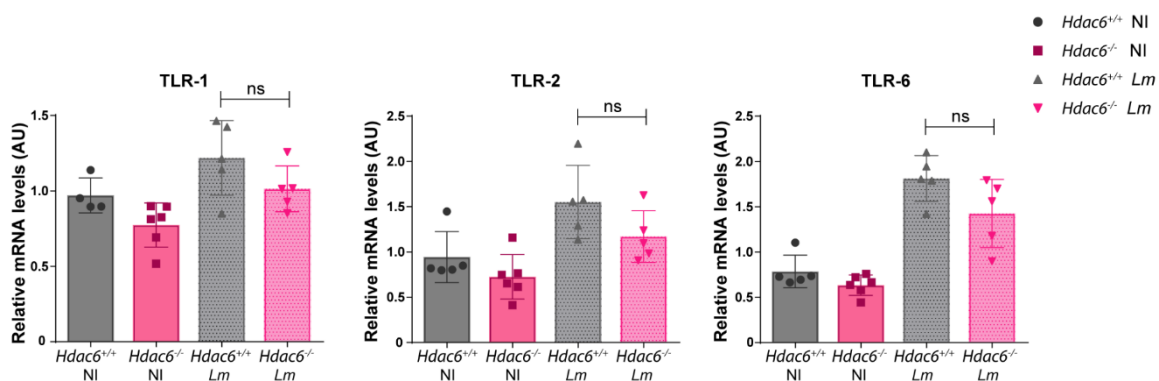


We next examined the effect of HDAC6 deficiency on TLR-signalling pathways using other TLR stimuli, including HKLM and LPS. AKT phosphorylation in *Hdac6*<sup>-/-</sup> DCs was decreased after LPS or HKLM treatment compared to *Hdac6*<sup>+/+</sup>, confirming defective TLR activation (**Figure 4.34. A and B**).



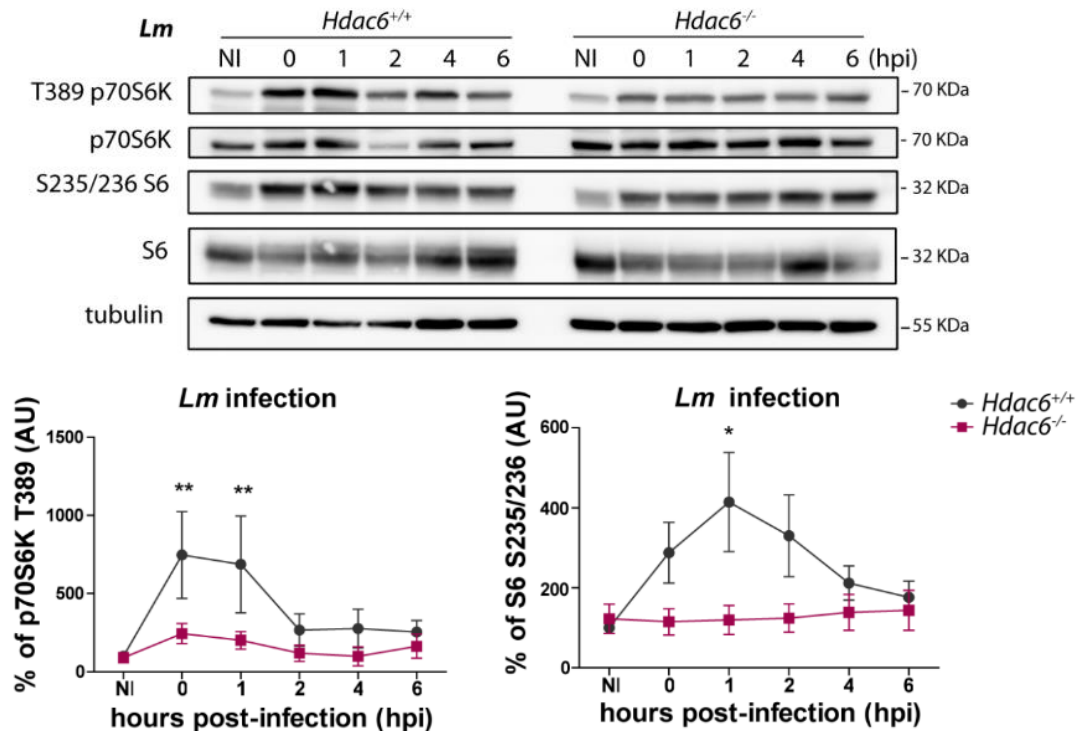
**Figure 4.34. Induction of AKT phosphorylation in BMDCs after stimuli with HKLM and LPS.** Left: Western-blot analysis in *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> DCs over the time-course of (A) HKLM or (B) LPS treatment. Total and phosphorylated AKT were detected for both treatments. Right: Accompanying charts on the right show quantification, indicating the percentage of pAKT/total AKT ratio. Two-tailed t-test, \*\*  $p \leq 0.01$ , \*  $p \leq 0.05$ ;  $n = 4$ .

These effects are not related to a defect in *Lm*-induced transcriptional induction since mRNA levels of different *Lm*-related TLRs (TLR1, 2, and 6) were similar in *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> GM-CSF DCs (**Figure 4.35**).



**Figure 4.35. mRNA expression of TLRs involved in the detection of *Lm* PAMPs.** RT-q-PCR analysis of TLR-1, 2 and 6 (AU: arbitrary units) in *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> DCs non-infected (NI) and after *Lm*-infection at 6 hpi. Two-tailed t-test, ns > 0.05 non-significant;  $n = 6$ .

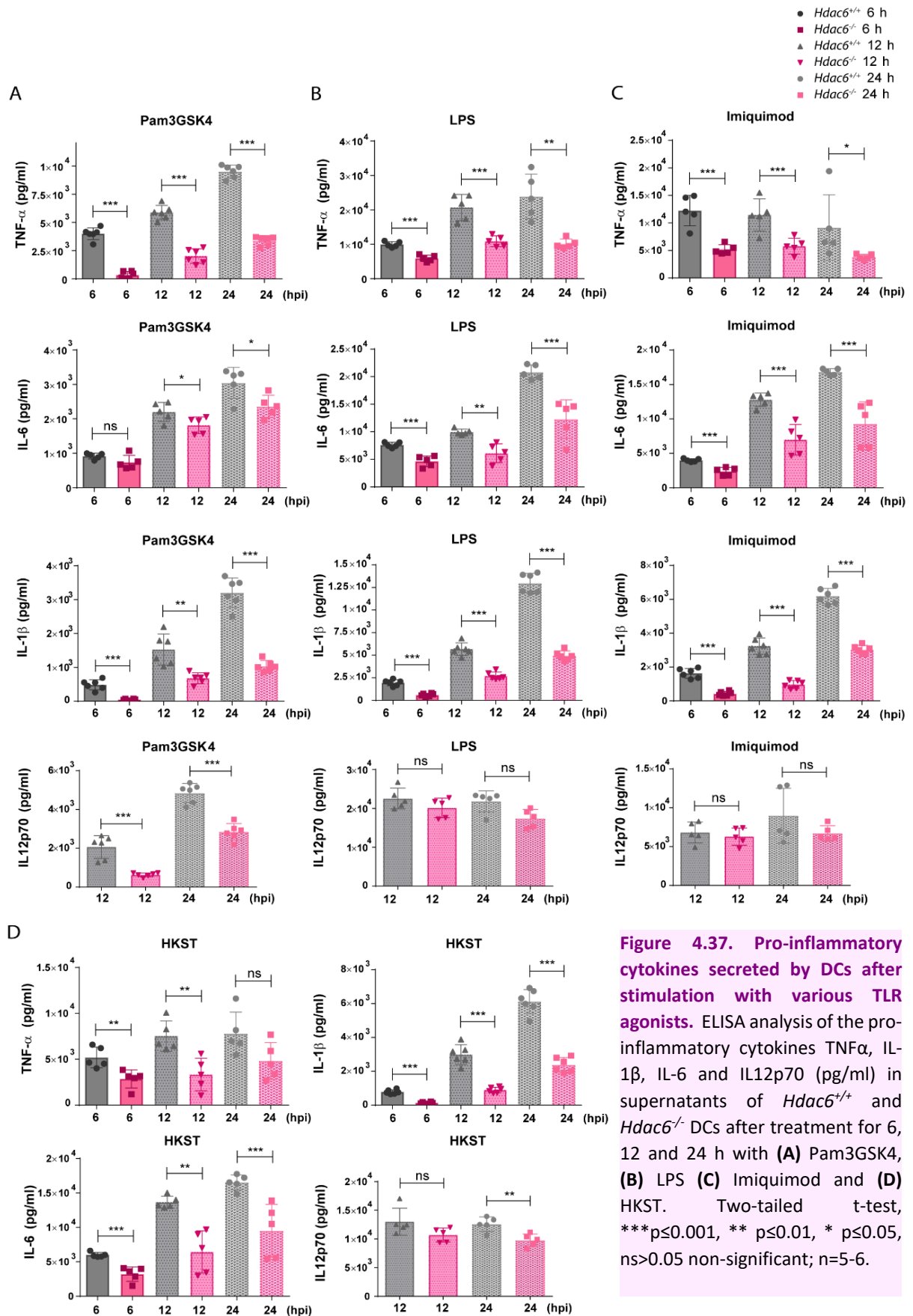
Moreover, *Hdac6*<sup>-/-</sup> DCs showed weaker phosphorylation of mammalian target of rapamycin complex 1 (mTORC1) pathway proteins (mTORC1 downstream substrates p70S6K and S6), consistent with a less pronounced pro-inflammatory response after TLR-activation by pathogen-associated molecular patterns (PAMPs) (**Figure 4.36**).



**Figure 4.36. mTOR activation after *Lm* infection.** Top panel: Western-blot analysis of mTORC1 pathway activation over the time-course of *Lm* infection in *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> DCs. Levels of phosphorylated and total p70S6K and S6 were detected. Tubulin was used as a loading control. Lower panel: Accompanying charts show quantification of p70S6K/total70S6K (n=5) and S6/totalS6 (n=7) ratios relative to the loading control. Two-tailed t-test, \*\* p≤0.01, ns non-significant.

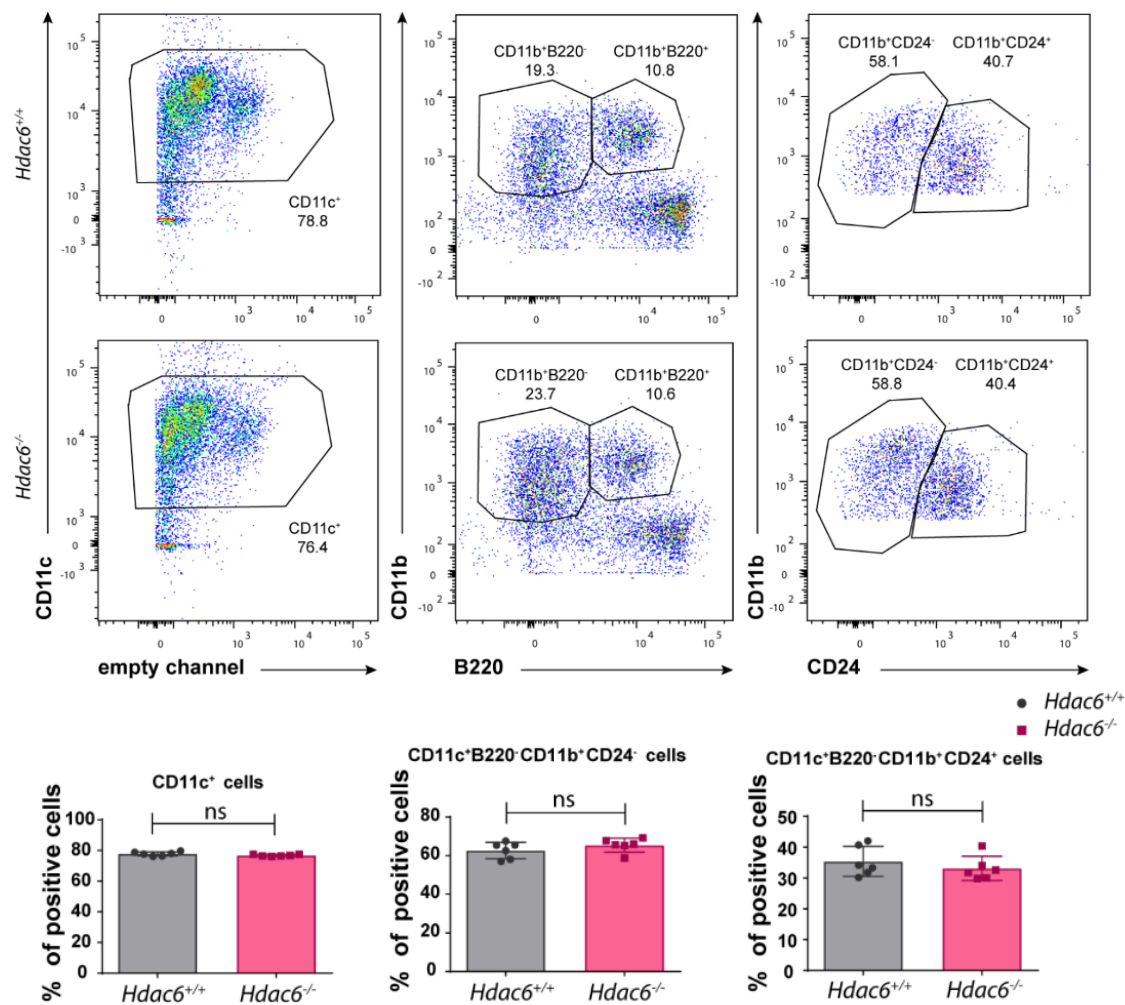
#### 4.2.6. Pro-inflammatory cytokine secretion after stimulation with TLR agonists

To determine whether *Hdac6*<sup>-/-</sup> DCs showed a similar defective response to other TLR agonists, we first examined secretion of pro-inflammatory cytokines in response to agonists of TLR1-2 (Pam3GSK4), TLR-4 (LPS), TLR-7-9 (Imiquimod), and multiple TLRs (heat-killed *Salmonella* Typhimurium; HKST). *Hdac6*<sup>-/-</sup> DCs showed a defective cytokine response to these stimuli, determined from the release of TNFα, IL-6, IL-1β and IL12p70 (**Figure 4.37. A-D**).



**Figure 4.37. Pro-inflammatory cytokines secreted by DCs after stimulation with various TLR agonists.** ELISA analysis of the pro-inflammatory cytokines TNF $\alpha$ , IL-1 $\beta$ , IL-6 and IL12p70 (pg/ml) in supernatants of *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> DCs after treatment for 6, 12 and 24 h with (A) Pam3GSK4, (B) LPS (C) Imiquimod and (D) HKST. Two-tailed t-test, \*\*\* $p$ ≤0.001, \*\*  $p$ ≤0.01, \*  $p$ ≤0.05, ns>0.05 non-significant; n=5-6.

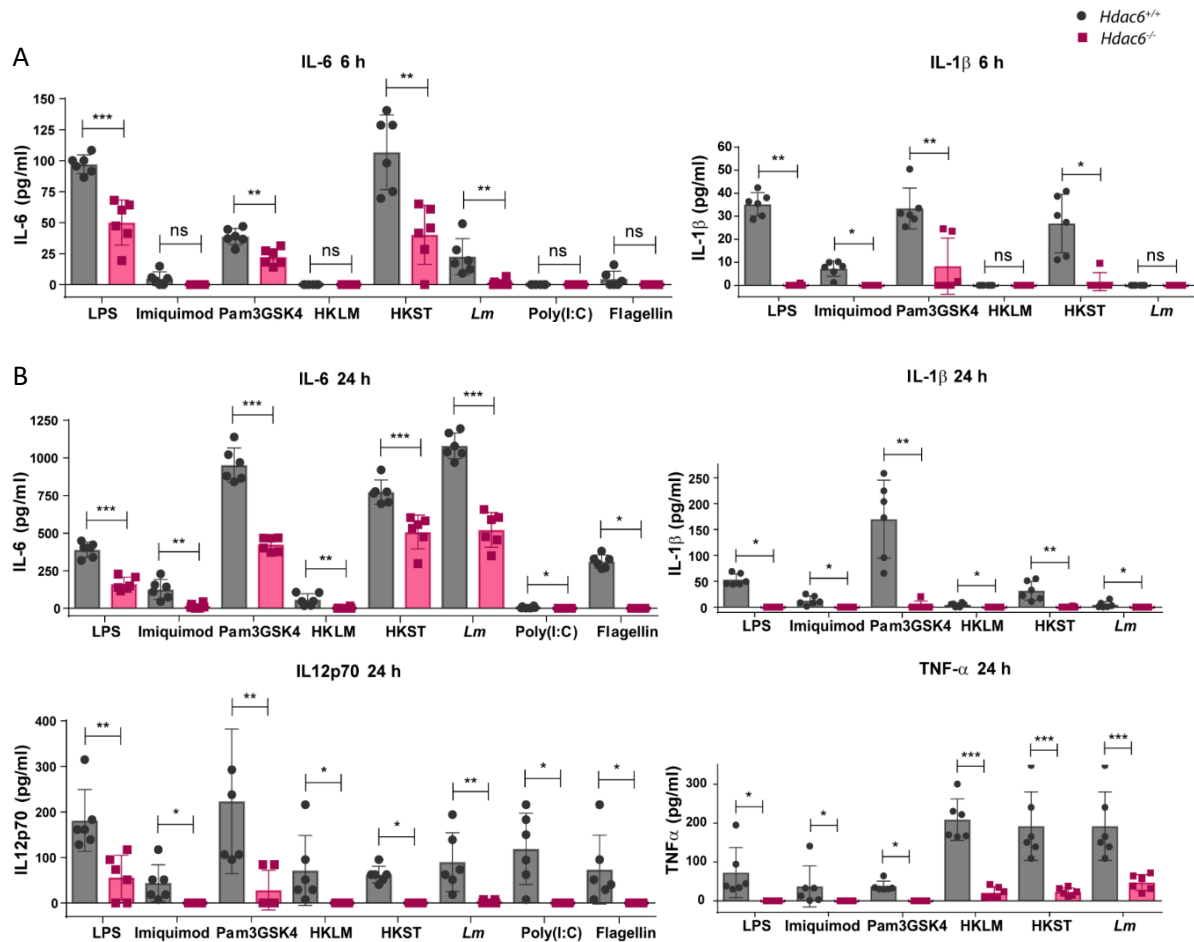
To assess the pro-inflammatory cytokine response to TLR3 and TLR5 ligands, we generated Fms-related tyrosine kinase 3 ligand-derived dendritic cells (FLT3L-derived DCs). Differentiation with the cytokine FLT3L yielded similar percentages of CD24<sup>+</sup> and CD24<sup>-</sup> subpopulations (CD11c<sup>+</sup>CD11b<sup>+</sup>B220<sup>-</sup> CD24<sup>+</sup> and CD11c<sup>+</sup>CD11b<sup>+</sup>B220<sup>-</sup>CD24<sup>-</sup>, respectively) from *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> DCs, indicating that differentiation is unaffected by HDAC6 absence (**Figure 4.38**).



**Figure 4.38. Characterization of FLT3L-derived DCs at day 11 of differentiation.** Left: Dot-plots of FLT3L-derived DC cultures at day 11 of differentiation, showing gating for the CD11c<sup>+</sup> population (percentages indicated). Centre: Dot-plots showing CD11b versus B220 to select two populations: CD11c<sup>+</sup>CD11b<sup>+</sup>B220<sup>+</sup> (plasmacytoid DCs, pDCs) and CD11c<sup>+</sup>CD11b<sup>+</sup>B220<sup>-</sup> (conventional DCs, cDCs) (percentages indicated). Right: Dot-plots showing CD11b versus CD24 to select the CD11b<sup>+</sup>CD24<sup>+</sup> and CD11b<sup>+</sup>CD24<sup>-</sup> populations (gated from cDCs) (percentages indicated). The charts on the right show the percentages of CD11c<sup>+</sup>, CD11c<sup>+</sup>CD11b<sup>+</sup>B220<sup>-</sup>CD24<sup>-</sup> and CD11c<sup>+</sup>CD11b<sup>+</sup>B220<sup>-</sup>CD24<sup>+</sup> populations. Two-tailed t-test, ns>0.05 non-significant; n=6.

The TLR agonists Pam3GSK4 (TLR1-2), Poly(I:C) (TLR3), LPS (TLR4), flagellin (TLR5), Imiquimod (TLR-7-9), *Lm*, HKLM, and HKST (which activates several TLRs simultaneously) elicited similar cytokine secretion profiles in GM-CSF DCs and FLT3L-DCs (**Figure 4.39. A** and **Figure 4.39. B** compared to **Figure**

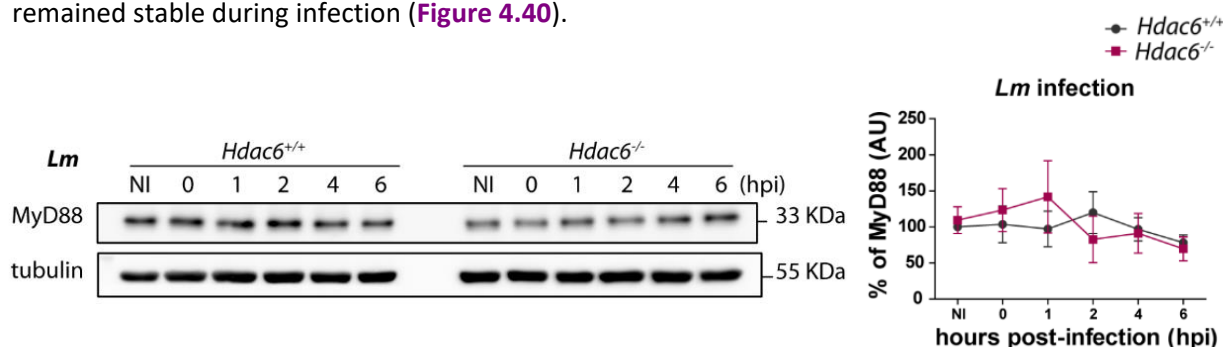
**4.37. A-D).** *Hdac6*<sup>-/-</sup> DCs of both origins showed an impaired cytokine response to each TLR agonist, indicating that HDAC6 likely regulates a common TLR signalling adaptor.



**Figure 4.39. Pro-inflammatory cytokines IL-6, IL-1β, IL-12p70 and TNF-α secreted by FLT3L-derived DCs after the stimulation with various TLR agonist.** ELISA detection of the pro-inflammatory cytokines IL-6, IL-1β, IL-12p70 and TNF-α (pg/ml) in supernatants of *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> FLT3L-derived DCs activated with LPS, Imiquimod, Pam3GSK4, HKLM, HKST, *Lm*, Poly(I:C) or flagellin for (A) 6 h and (B) 24 h. Two-tailed t-test, \*\*\*p≤0.001, \*\* p≤0.01, \* p≤0.05; n=6.

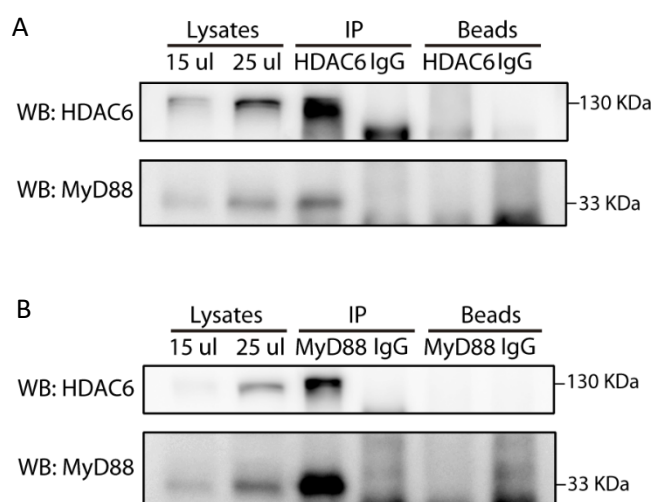
#### 4.2.7. Consequence of HDAC6 defect in the activation of TLR-signalling pathway

In this view, the TLR adaptor MyD88 participates in the transmission of signals by all TLRs except for TLR3. We decided to study MyD88 levels in a time-course infection with *Lm* by western blot, demonstrating that the quantity of this molecule was the same in *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> DCs and remained stable during infection (**Figure 4.40**).



**Figure 4.40. Levels of MyD88 adaptor protein during *Lm* infection.** Left: Western-blot analysis of MyD88 protein level over the time-course of *Lm* infection in *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> DCs. Tubulin was used as a load control. NI correspond to non-infected cells and infected cells are displayed as 0, 1, 2, 4 and 6 hpi. Right: Accompanying charts show quantification of the percentage of MyD88. MyD88 levels were normalized with its corresponding tubulin and expressed as percentage in arbitrary units. NI sample is considered as 100% of signal and the rest of samples are relativized to it. Two-tailed t-test, ns non-significant; n=5.

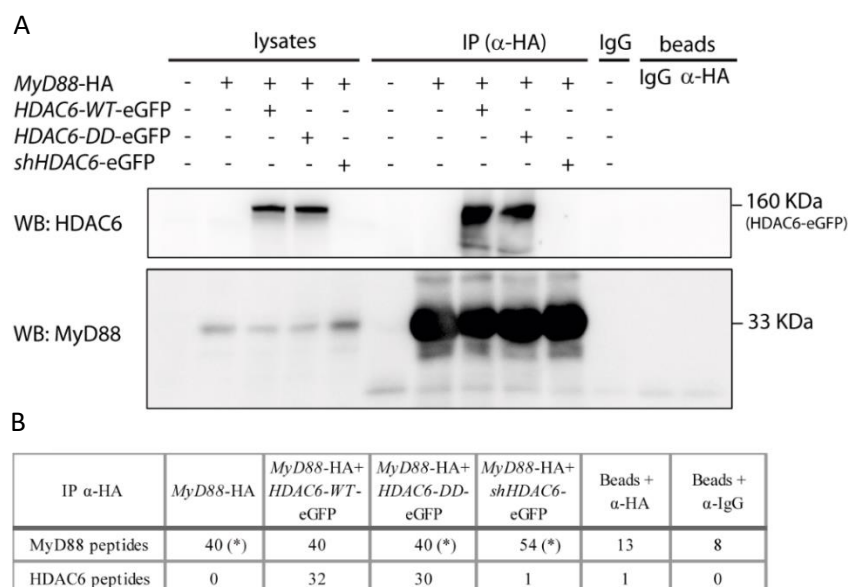
Remarkably, MyD88-HDAC6 molecular association was observed by co-immunoprecipitations of endogenous proteins using human dendritic cells after Pam2GSK4, Pam3GSK4 and HKLM stimulation (**Figure 4.41**).



**Figure 4.41. Molecular association of HDAC6 with the MyD88 adaptor protein.** Immunoprecipitation of endogenous (A) HDAC6 and (B) MyD88 followed by western-blot for both proteins. Immunoprecipitations were carried out using human moDCs after 30 min of stimulation with Pam2GSK4, Pam3GSK4 and HKLM. Molecular weight of endogenous HDAC6 (130 kDa) and MyD88 (33 kDa) are indicated at the right of western-blot. Similar results were obtained in two independent experiments.

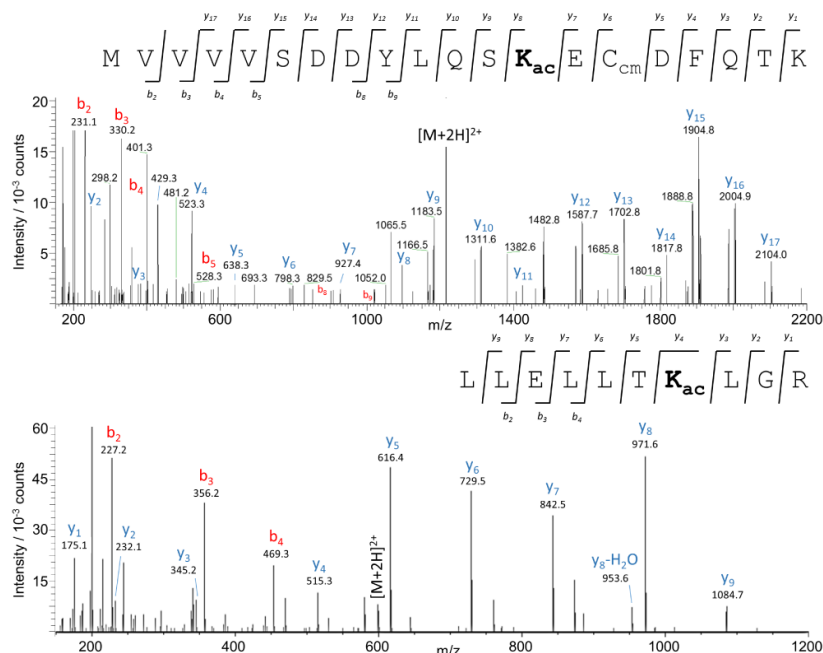
Likewise, the MyD88 immunoprecipitation in *MyD88*- and *HDAC6*-overexpressing HEK cells was also able to co-precipitate HDAC6 (**Figure 4.42**).





**Figure 4.42. Molecular association of HDAC6 and MyD88 adaptor protein. (A)** Immunoprecipitation of HA (MyD88) followed by western-blot for HDAC6 and MyD88. Immunoprecipitations were carried out using different *HDAC6-eGFP* plasmids co-transfected with *MyD88-HA* in HEK cell line. Over-expressed (HDAC6-eGFP, 160 kDa) is indicated at right of western-blot. Similar results were obtained in three independent experiments. **(B)** Immunoprecipitation of HA (MyD88) followed by mass spectrometry analysis. Immunoprecipitations were carried out using different *HDAC6-eGFP* plasmids co-transfected with *MyD88-HA* in HEK cell line. The number of unique MyD88 and HDAC6 peptides identified is indicated. (\*) indicates the presence of acetylated MyD88 peptides. Similar results were obtained in three independent experiments.

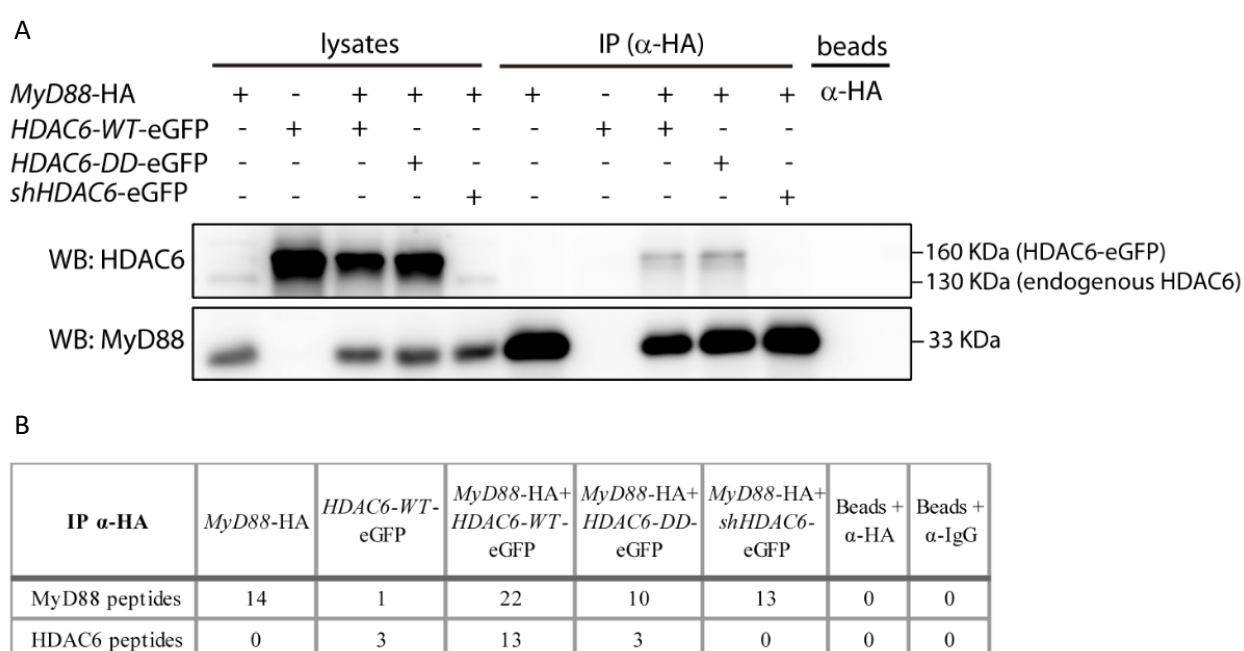
These results were corroborated by mass spectrometry analysis of MyD88 immunoprecipitates; which in addition detected two acetylated peptides corresponding to MyD88 (**Figure 4.42. B** and **Figure 4.43**).



**Figure 4.43. Acetylated lysines on MyD88 adaptor protein.** MS<sup>2</sup> fragmentation spectra from the peptides showing at 1217.0699 (Top), and 599.3803 (Bottom). Ion adscription to carboxy- (y ions, blue) and amino-terminal (b ions, red) fragmentation series is indicated.  $K_{ac}$  denotes acetylated lysine and  $C_{cm}$  indicates carbamidomethylated cysteine. Fragment ion sequence coverage is schematically indicated. Similar results were obtained in three independent experiments.

The same approach was used to determine MyD88-HDAC6 molecular association after TLR-2 activation with HKLM using a constitutively expressed human TLR-2 HEK cell line, rendering the same result (**Figure 4.44**). This association is also maintained using a double-deacetylase domain mutant of HDAC6 (H216A:H611A), called *HDAC6-DD*, indicating that HDAC6-MyD88 interaction is independent of its catalytic activity (**Figure 4.42** and **Figure 4.44**). The knock-down of HDAC6 expression using a small hairpin plasmid (*sh-HDAC6*) blocked this interaction (**Figure 4.42** and **Figure 4.44**).

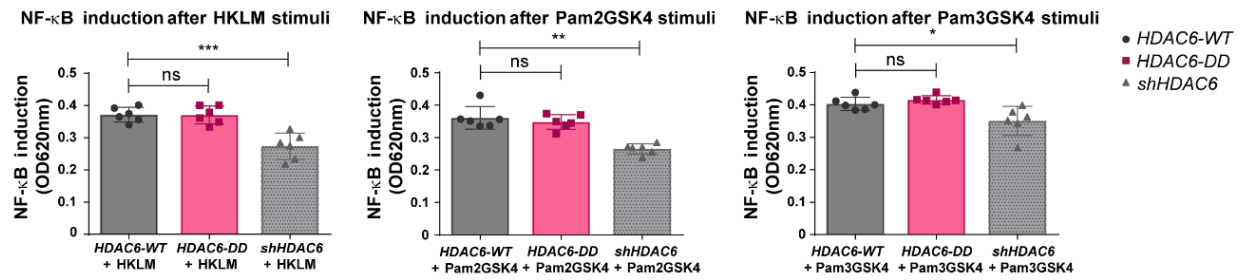
However, no acetylated peptides could be detected in the mass spectrometry analysis of the MyD88 immunoprecipitation from HKLM-stimulated TLR-2 HEK cell line (**Figure 4.44. B**).



**Figure 4.44. Molecular interaction of HDAC6 with the MyD88 adaptor protein. (A)** Immunoprecipitation of HA (MyD88) followed by western-blot for HDAC6 and MyD88. Immunoprecipitations were carried out using different *HDAC6-eGFP* plasmids co-transfected with *MyD88-HA* in HEK-Blue hTLR2 cell line after 30 min of stimulation with HKLM. Over-expressed (HDAC6-eGFP, 160 KDa) and endogenous HDAC6 (130 KDa) are indicated at right of western-blot. Similar results were obtained in four independent experiments. **(B)** Immunoprecipitation of HA (MyD88) followed by mass spectrometry analysis. Immunoprecipitations were carried out using different *HDAC6-eGFP* plasmids co-transfected with *MyD88-HA* in HEK-Blue hTLR2 cell line after 30 min of stimulation with HKLM. The number of unique MyD88 and HDAC6 peptides is indicated. No acetylated peptides from MyD88 were detected in any sample. Similar results were obtained in four independent experiments.

Moreover, assessment of Nuclear factor- $\kappa$ B (NF- $\kappa$ B) induction in TLR-2-expressing HEK cells after HKLM, Pam2GSK4 and Pam3GSK4 stimulation shows lower activation only in *shHDAC6* transfected cells, without affecting the activity of *HDAC6-DD*-transfected cells (**Figure 4.45**).



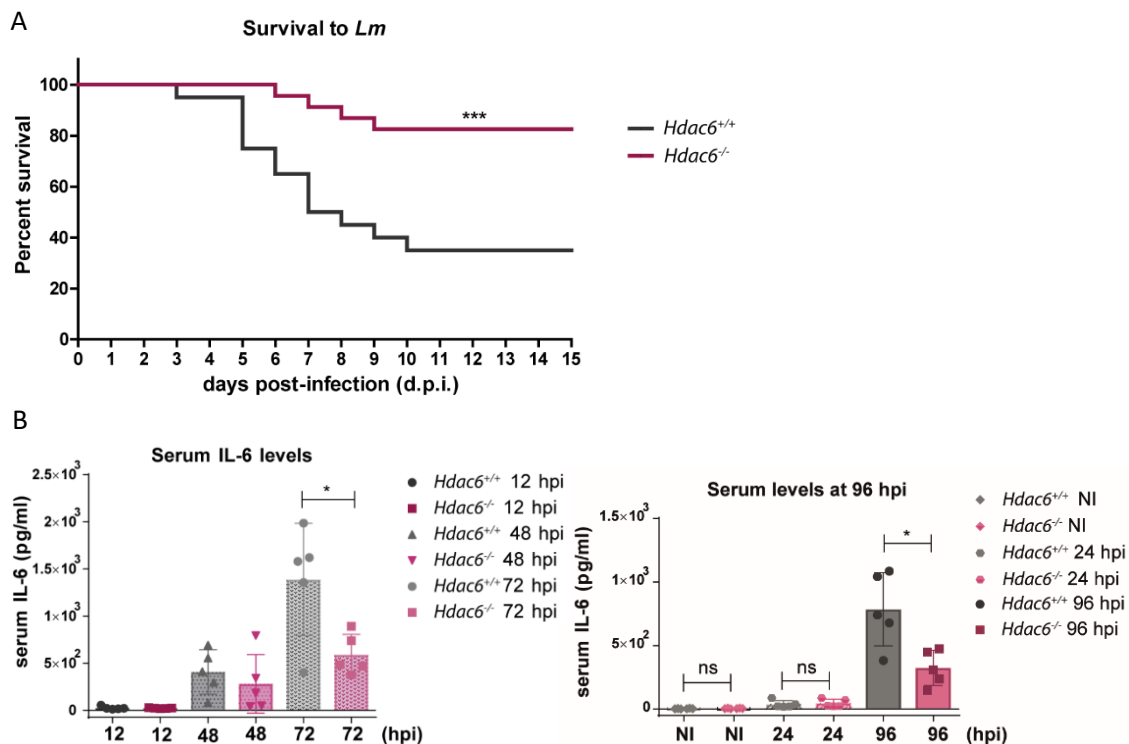


**Figure 4.45. NF-κB induction after TLR-2 activation.** Graph of NF-κB induction in transfected *HDAC6-WT*, *HDAC6-DD* and *shHDAC6* HEK-Blue hTLR2 cell line after activation with HKLM, Pam2GSK4 and Pam3GSK4 during 8 h. NF-κB induction was calculated by the ratio of the signal of stimulated cells with its corresponding transfected cells in basal condition (without stimuli). Two-tailed t-test, \*\*\* $p \leq 0.001$ , \*\* $p \leq 0.01$ , \* $p \leq 0.05$ , ns>0.05 non-significant; n=6.

Taking into account all these data, we concluded that HDAC6 associates with the TLR-adaptor molecule MyD88, and the absence of HDAC6 in DCs seems to diminish the TLR-response after a variety of stimuli, underlining the scaffold role of HDAC6 in determining the ability of MyD88 to mediate TLR signalling.

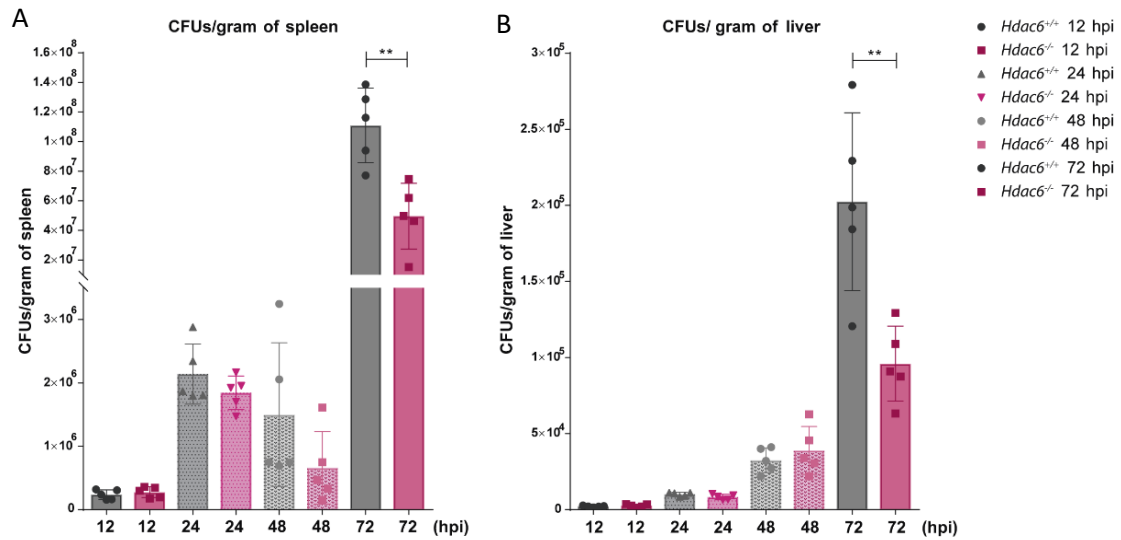
#### 4.2.8. Evaluation of the HDAC6 role in the *in vivo* TLR-dependent inflammatory response

To ascertain the defective TLR-dependent inflammatory response *in vivo* due to the molecular MyD88-HDAC6 association, *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> mice were intravenously injected with a lethal dose of *Lm* (Figure 4.46. A) [150]. A protective effect against *Lm*-induced septic shock was observed from 3 to 10 days post-infection (dpi) (Figure 4.46. A). Accordingly, lower levels of the pro-inflammatory cytokine IL-6 were detected in the serum of *Hdac6*<sup>-/-</sup> mice at 72 hpi, highlighting a reduced systemic cytokine-driven inflammatory response after *Lm* infection in these mice (Figure 4.46. B).



**Figure 4.46. Inflammatory response of *Hdac6*<sup>-/-</sup> mice to *Lm*.** (A) Survival curve to intravenous injection with a lethal dose of *Lm* in *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> is showed. This curve corresponds to two different experiments of survival to *Lm* with a n=24-21. Long-rank (Mantel-Cox) test and Cehan-Breslow-Wilcoxon test, \*\*\*p<0.001. (B) Pro-inflammatory cytokine IL-6 was measured in sera of *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> mice intravenously injected with a lethal dose of *Lm* at 12, 24, 48, 72 and 96 hpi and NI (non-infected). Two-tailed t-test, \*p<0.05, n=5.

No significant differences in spleen and liver bacterial load were found between *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> from 12 to 48 hpi (Figure 4.47). However, higher bacterial burden was distinguished in target organs of *Lm*-induced septic shock *Hdac6*<sup>+/+</sup> mice at 72 hpi (Figure 4.47). This observation is in accordance with the increased IL-6 level in *Hdac6*<sup>+/+</sup> mice sera at 72 hpi, suggesting an impairment of the anti-bacterial clearance response in these animals (Figure 4.47 and compared with Figure 4.46. B).



**Figure 4.47. Bacterial burden in target organs of *Hdac6*<sup>-/-</sup> mice.** Quantification of bacterial load in target organs, **(A)** spleen and **(B)** liver, at 12, 24, 48 and 72 hpi in *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> mice injected with a lethal dose of *Lm*. Bacterial load is expressed by CFUs per gram of spleen and per gram of liver. Two-tailed t-test, \*\*p<0.01, n=6.



# Discussion



## 5. DISCUSSION

Here we present evidence suggesting a critical role for HDAC6 in the regulation of innate immune responses. Our results underscore a fundamental role of HDAC6 in almost every step of dendritic cell functions against the intracellular bacteria *Lm*, starting with the control of bacterial degradation by autophagy, cytokine production and regulation of the outcome of anti-bacterial host response. By regulating the fusion of autophagosomes with lysosomes, HDAC6 is able to degrade invading pathogens and reduce bacterial burden, which is critical to efficiently initiate the signalling pathways involved in anti-bacterial response. In addition, our results on the functional regulation of HDAC6 during TLR stimulation of pro-inflammatory cytokine production after *Lm* infection demonstrate the scaffold role of HDAC6 in the TLR-signalling pathway as a MyD88-interacting protein, providing a novel role of HDAC6 in the activation of innate immune responses against intracellular bacteria infection. Finally, we present evidence indicating a major role of HDAC6 in the context of pro-inflammatory cytokine production, which is associated with the exacerbated inflammatory cytokine storm found during septic shock caused by *Gram*-positive bacteria. These results open a new field for the application of HDAC6 specific inhibitors in septic shock cases to avoid exacerbated inflammation.

### 5.1. Analysis of HDAC6 contribution to cellular mechanisms that eliminate intracellular bacteria by phagocytic cells

Recent studies have revealed the involvement of HDAC6 in the innate immune response against Influenza Virus A (IVA), Sendai virus (SeV), and vesicular stomatitis virus (VSV) [243,271,15,93]. Given the similarities between the innate responses to viruses and intracellular bacteria, this prompted us to investigate the role of HDAC6 in a model of *Listeria monocytogenes* infection. In this work, we demonstrate a dual role of HDAC6 in the innate response against *Lm*, not only due to its enzymatic activity but also dependent of its function as a scaffold protein (Figure 5.1 and Figure 5.3).

#### 5.1.1. Effect of HDAC6 absence in the intracellular pathogen clearance of dendritic cells

Our results describe an internalization up to 20 bacteria per DC as it has been previously described in phagocytic cells [195]. The significant median bacterial uptake by GM-CSF DCs was observed in cases of 6-7 bacteria per cell, although the distribution of the number of phagocytosed bacteria per cell indicates that *Hdac6*<sup>-/-</sup> DCs display higher *Lm* titres than *Hdac6*<sup>+/+</sup> DCs independently of the number of bacteria per cell. Our data clearly demonstrate that *Hdac6*<sup>-/-</sup> DCs have an impaired immune response against *Lm* and *S. Typhimurium* infection *in vitro*. Moreover, higher *Lm* titres observed in *Hdac6*<sup>-/-</sup> DCs,

M-CSF-derived macrophages and peritoneal macrophages were corroborated during *in vivo* *Lm* infection at 6 hpi in various myeloid subsets of the spleen, including monocytes, neutrophils, cDCs, and TipDCs. The absence of this effect during DC *in vitro* infection by the non-intracellular bacteria *S. aureus* and *E. coli* DH5 $\alpha$  indicates that *Hdac6*<sup>-/-</sup> DCs are specifically unable to efficiently clear intracellular pathogens.

### 5.1.2. Evaluation of cellular clearance mechanisms of *Lm*

Intracellular pathogens have developed specialized tools to evade cellular clearance mechanisms. For instance, *S. Typhimurium* uses a type-III secretion system to escape from *Salmonella*-containing vacuoles (SCVs), whereas *Lm* uses the pore-forming enzyme LLO and two phospholipases, PlcA and PlcB, to reach the host cell cytosol.

Additionally, HDAC6 is involved in two of the most important cellular clearance systems, autophagy and ubiquitin-proteasome pathway (UPP) [127,186]. In the case of *Lm* and *S. Typhimurium*, the main molecular mechanism for degradation of vesicle-contained bacteria is fusion with lysosomes in a process called autophagy [205,197,24]. In agreement with this, our data show that impaired phagosome-lysosome fusion underlies the phenotype observed in *Hdac6*<sup>-/-</sup> DCs.

It has been previously reported that unsuccessful phago-lysosomal fusion depends on acetylated-cortactin in *Hdac6*-deficient cells [127]. This mechanism was first described in *Hdac6*-deficient mouse embryonic fibroblasts (MEFs) during quality-control autophagy [127]. Quality-control autophagy is a selective autophagy as well as the specific autophagy of *Lm* that we have observed in this work, which is commonly known as xenophagy. We demonstrate here that in *Hdac6*<sup>-/-</sup> DCs co-localize higher levels of acetylated-cortactin with intracellular *Lm*. The delay in vesicle fusion caused by the acetylation of cortactin, impairs the phagosome-lysosome fusion and provides more opportunities for phagosome-containing bacteria to escape to the cytosol, resulting in the higher bacterial load detected in *Hdac6*<sup>-/-</sup> DCs. Based on this experimental evidence, it is conceivable to postulate that the enzymatic activity of HDAC6 on its substrate cortactin controls autophagy of intracellular bacteria for their efficient clearance (Figure 5.1 part 1 and Figure 5.3 part 1). Hence, *Hdac6*<sup>-/-</sup> DCs displays higher levels of acetylated cortactin and consequently more *Lm* burden.

### 5.1.3. Consequence of the lack of HDAC6 in the autophagy of *Lm*

Pharmacological autophagy inhibitors erased the observed differences between *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> DCs, remarking the impaired autophagy detected in *Hdac6*<sup>-/-</sup> DCs. Conversely, rapamycin did not overcome the *Hdac6*<sup>-/-</sup> autophagy defect. Rapamycin inhibits mTOR activity, causing an increment of the autophagic flux. Increased p62 levels were observed after rapamycin treatment, although *Hdac6*<sup>-/-</sup>



<sup>-/-</sup> DCs accumulated higher p62 levels at 6 hpi compared to *Hdac6*<sup>+/+</sup> DCs, indicating a defect in the last step to autophagy, the phagosome-lysosome fusion. Accordingly, similar results were obtained after the treatment with inhibitors of acidification of lysosomal enzymes, such as NH<sub>4</sub>Cl and cloroquine, further supporting this observation. However, other authors have reported opposite observations using pan-HDAC inhibitors (HDACi) or specific inhibitors of HDAC6 (HDAC6i) during infection of human macrophages with the *Gram*-negative intracellular pathogens *S. Typhimurium* and *E. coli* [5]. These inhibitors, when added at the time of infection, increase mitochondrial ROS production [5]. Overnight pre-treatment with HDACi before infection hampers bacterial clearance and reduces phagocytosis [5]. These data indicate that specific HDAC6 chemical inhibitors can have side-effects, including effects on other HDAC members, potentially interfering with the acetylation of other substrates upstream of cortactin that also have a role during bacterial infection. We have not observed any significant difference during bacterial entry between *Hdac6*<sup>-/-</sup> and *Hdac6*<sup>+/-</sup> DCs. Results obtained after overnight pre-treatment with HDAC6i in phagocytosis indicate additional acetylations on cytoskeleton proteins that do not occur in *Hdac6*<sup>-/-</sup> DCs. Hence, our genetic approach unequivocally assigns a specific role to HDAC6 in innate cells during bacterial infection.

#### 5.1.4. Effect of HDAC6 absence in the accumulation of autophagy marker p62

Although we observed an impairment in phagosome-lysosome fusion, we cannot rule out an involvement of HDAC6 in the anti-microbial response through its BUZ domain, with a contribution from ubiquitin. The characterized interaction between p62 and HDAC6 through their ubiquitin-binding domains provides a clue about the possible role of ubiquitin in the activation of innate immunity through the recognition of ubiquitinated-molecules [262]. For example, the ubiquitin-binding regions of HDAC6 and p62 are both essential for MyD88 aggregation and the downstream activation of MyD88-dependent signal transduction [97]. Furthermore, ubiquitin-binding platforms formed by HDAC6 and p62 are able to interact with interferon stimulated gene 15 (ISG15) to eliminate ISGylated proteins tagged after interferon stimulation by autophagy [166]. These ubiquitin-binding platforms also bind protein aggregates tagged by lysine 63 (K63)-linked polyubiquitin (polyUb) chains and degrade them by autophagy. HDAC6 is able to bind to either mono- and poly-Ub proteins, but shows a preference for proteins modified with K63-linked polyUb chains, which share structural similarities with ISG15 [180,179]. *S. Typhimurium* is decorated with this kind of ubiquitin topology for recognition by host cells, and can be recovered with phagosome proteins to initiate autophagy [210]. Nevertheless, we were not able to detect K63 polyUb signal at early times of infection in our *Lm*-infected DC samples (data not shown). Consequently, our data demonstrate that autophagy induction does not differ between *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> DCs, indicating that this phenotype is due to p62 accumulation in

*Hdac6*<sup>-/-</sup> BMDCs as a consequence of impaired phagosome-lysosome fusion. Intact autophagy activation in *Hdac6*<sup>-/-</sup> DCs could be explained by compensatory p62 binding to ubiquitinated bacteria in the absence of HDAC6. However, higher K63 polyUb signal detected in *Hdac6*<sup>-/-</sup> DCs at late time of infection (about 6 hpi) is clearly in accordance with the observation of higher p62 levels co-localizing with *Lm* in these DCs. Besides, it has been previously reported that an impaired autophagy results in the accumulation of the p62 autophagy marker and the subsequent increased amount of polyUb proteins. Together, these data indicates that HDAC6-deficient cells display an altered accumulation of p62 and K63 polyUb proteins due to the impaired phagolysosomal fusion.

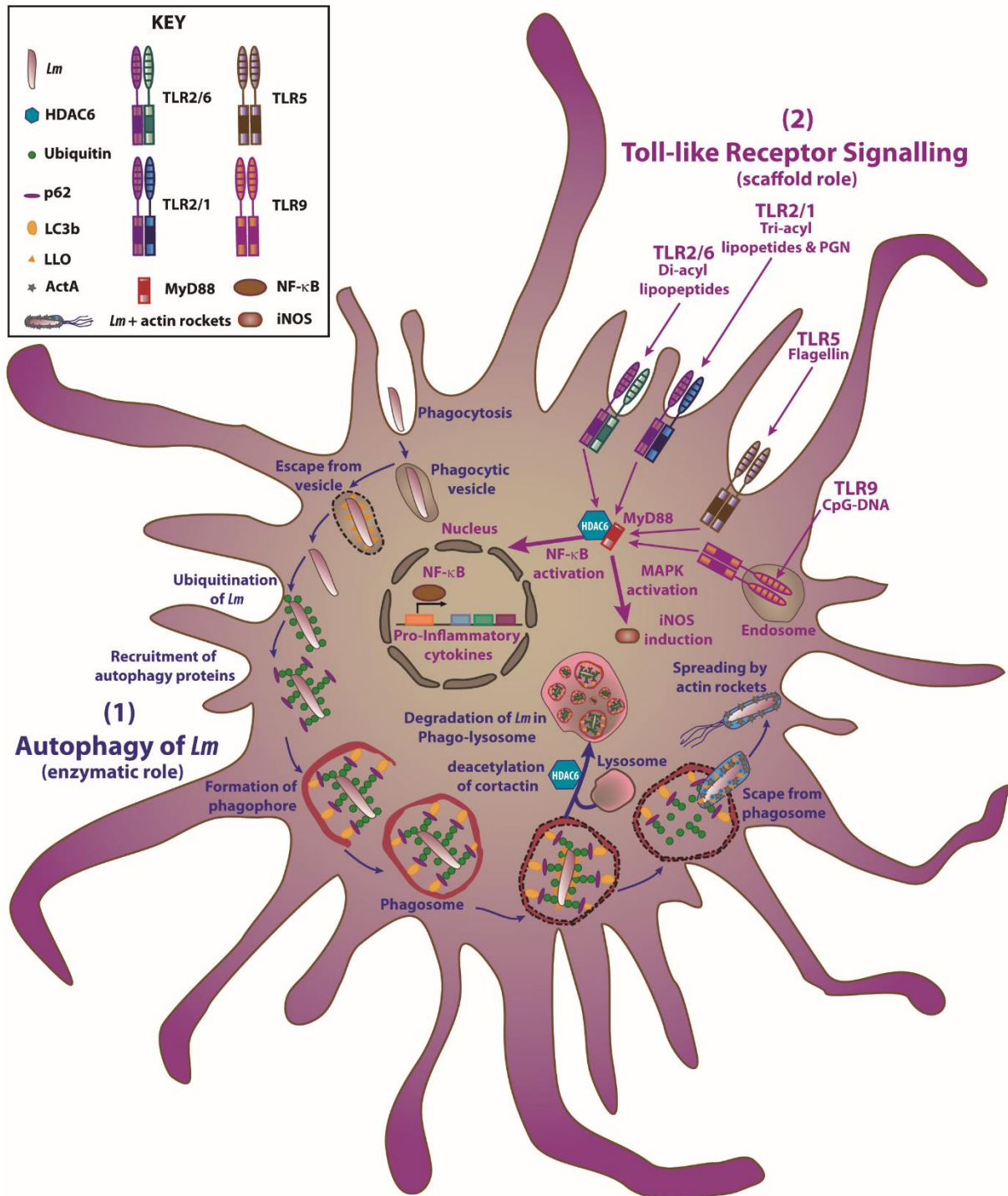
## **5.2. Study of the role of HDAC6 in the activation of dendritic cell functions by bacterial infection and stimulation of TLRs**

Our data also underscore another different function of HDAC6 in the innate immune response to intracellular bacteria and various TLR stimuli, independent of its enzymatic activity on cortactin (**Figure 5.1** and **Figure 5.3**). Upon infection, the activation of pro-inflammatory responses to alert immune system cells is an essential step for the elimination of intracellular pathogens.

### **5.2.1. Impaired inflammatory response**

We provide evidence for a dampened inflammatory response in the absence of HDAC6, as shown by lower RNA levels of pro-inflammatory cytokines, chemokines, type-I interferons, and interferon-related proteins in *Hdac6*<sup>-/-</sup> DCs than in *Hdac6*<sup>+/+</sup> cells at 6 hpi, as well as the lower pro-inflammatory cytokine production of IL-1 $\beta$ , IL-6, IL-12p70 and TNF- $\alpha$  by *Lm* infected *Hdac6*<sup>-/-</sup> DCs from 6 to 24 hpi. This global diminished production of pro-inflammatory cytokines could reflect a defect in the TLR-signalling pathway.

Whole pathogens and their constituents contain Pathogen-associated molecular patterns (PAMPs) that activate TLRs, NLRs and other cytosolic receptors in host cells [108,162]. These receptors recognize general molecular patterns of microbes to rapidly activate the immune response. Upon *Lm* entry, heterodimers of TLR-2/6 and TLR-1/2 recognize respectively di-acyl- and tri-acyl-lipopeptides of *Lm*. Then, a receptor-signalling complex is triggered upon TLR activation where signalling proteins are modified by PTMs such as phosphorylation and ubiquitination [234,198]. Both are reversible PTMs that allow transient protein-protein interactions occurring during signal transduction.



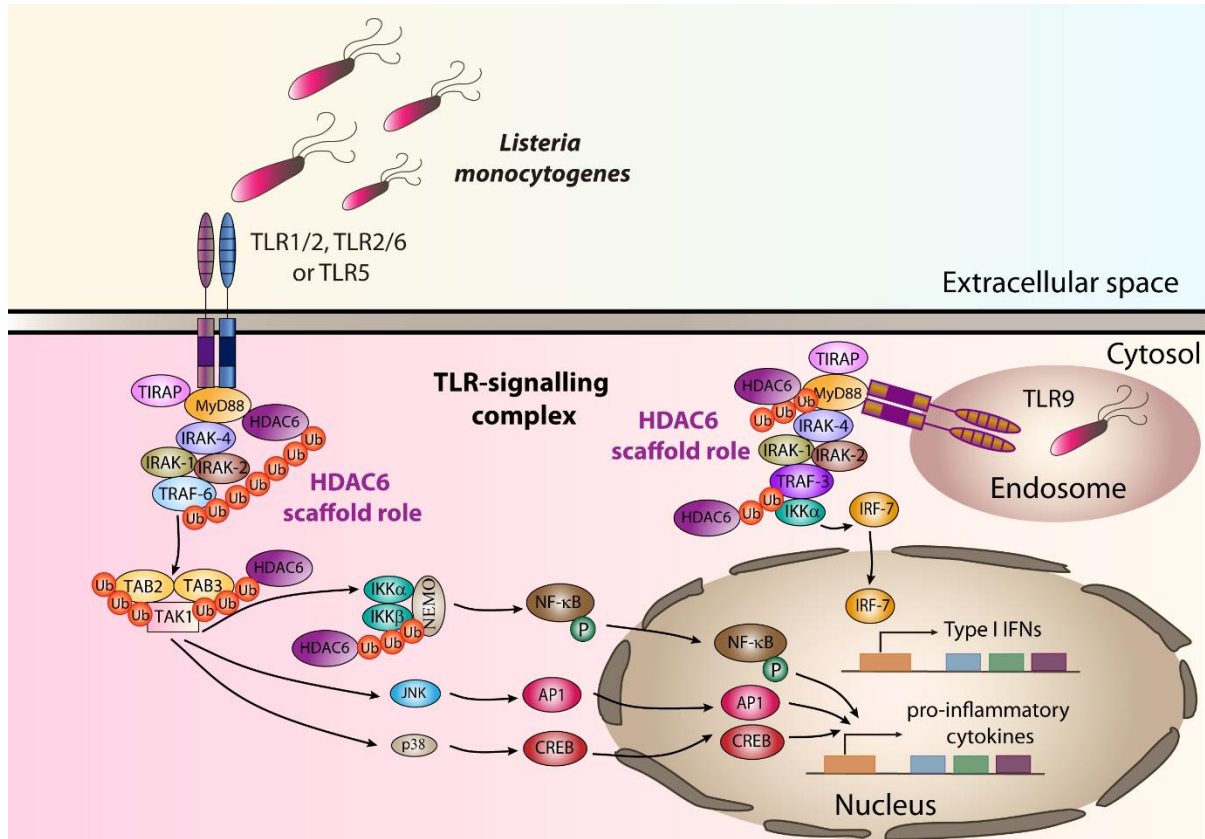
**Figure 5.1. Dual role of HDAC6 during *Lm* infection in DCs.** The figure shows two HDAC6 functions in *Lm*-infected DCs, the autophagy and the TLR signalling. **(1) Autophagy:** The deacetylation of cortactin by HDAC6 allows the correct phago-lysosomal fusion and the subsequent clearance of *Lm*. However, this bacterium is able to avoid autophagic degradation by the expression of LLO protein. Besides, *Lm* spread through the cytosol using ActA protein to polymerize cell host actin. **(2) TLR-signalling pathway:** Di- and tri-acyl lipopeptides and peptidoglycan (PGN) of *Lm* are recognized by TLR-2/6 and TLR-1/2, flagellin by TLR-5 and CpG DNA by TLR-9 upon *Lm* entry. HDAC6 interacts with the TLR-adaptor protein MyD88, enhancing down-stream TLR-signalling, (increasing NF-κB and MAPK activation). This stronger activation seems to be independent of HDAC6 enzymatic activity and causes higher pro-inflammatory cytokine production and more iNOS induction, necessary for the correct activation of anti-bacterial transcription program. Note that both processes occur during *Lm* infection and the pro-inflammatory cytokines and iNOS induction can impact on the autophagic process. The images in the figure are not scaled.

The TLR universal adaptor protein MyD88 is also activated, together with other adaptor protein called TIRAP (Toll/Interleukin-1 receptor domain containing adapter protein) or MAL (MyD88-adaptor-like), followed by Interleukin-1 receptor-associated kinases (IRAK) 1, 2 and 4, TNF receptor-associated factor 6 (TRAF-6), finally transducing nuclear signals through Activator Protein 1 (AP-1) and NF- $\kappa$ B TFs leading to pro-inflammatory cytokine secretion [172]. In this regard, the E3-ubiquitin protein ligase TRAF-6 catalyzes the formation of K63-linked polyUb chains and participates in the canonical NF- $\kappa$ B activation pathway, ubiquitinating Transforming growth factor- $\beta$  (TGF- $\beta$ ) Activated Kinase 1 (TAK1): TAK-1 binding proteins 1, 2 and 3 (TAB1-3) and NF- $\kappa$ B essential modulator (NEMO):I $\kappa$ B kinase (IKK) complexes [48,105,139]. The canonical IKK complex is formed by two kinases, IKK $\alpha$  and IKK $\beta$  and their regulatory protein NEMO, which has an ubiquitin-binding domain (UBD) that recognizes K63-linked polyUb chains, an essential step for the activation of the NF- $\kappa$ B pathway [64,258]. In this case, K63-Ub chains function as molecular scaffolds for the recruitment of both IKK and TAK1 complexes, enhancing the TAK1-dependent activation of the IKKs [64,258]. Hence, ubiquitination of proteins belonging to the TLR signalling complex can fine tune innate immune responses to pathogens [142].

It has been also described that the expression of HDAC6 in the nucleus deacetylates p65 subunit of NF- $\kappa$ B, reducing its capacity to bind the Matrix metalloproteinase 2 (MMP2) promoter thereby causing the inhibition of tumour invasion [263]. Hence, deacetylation of NF- $\kappa$ B by HDAC6 adds more complexity to signalling regulation. Moreover, a suppressed activation of NF- $\kappa$ B and AP-1 pathways has been observed in HDAC6-silenced cells after HIV-1 Tat-stimulation [226]. Moreover, *Lm*-derived lipoteichoic acids and LLO induce NF- $\kappa$ B signalling during infection, which seems to be MyD88-independent [87,109]. TAB2 and TAB3 are receptors which preferentially bind K63-linked polyUb chains through a highly conserved zinc finger domain (ZnF), very similar to the HDAC6 BUZ domain [105]. PolyUb-binding domains represent a new class of signaling structures that regulate protein kinase activity through a nonproteolytic mechanism. This issue may suggest a specific role of HDAC6 as an Ub-binding receptor in the NF- $\kappa$ B pathway, and this is in accordance with the lower phosphorylation observed in downstream MAPK signalling pathway in *Hdac6*<sup>-/-</sup> DCs. The Figure 5.2 summarizes the tentative model of HDAC6 regulation of MyD88-dependent TLR-signalling pathway during *Lm* infection.

Moreover, during TLR-signalling MAPKs are also activated and the microbicidal activity of iNOS is induced [108]. For instance, HIV-1 Tat-induced activation in HDAC6-silenced cells shows a reduced induction of MAPKs, including ERK, c-Jun N-terminal kinase (JNK) and p38 signalling pathways [226]. In fact, *Hdac6*<sup>-/-</sup> DCs show diminished iNOS induction at 6 hpi associated with low nitrite production and iNOS expression at longer times of *Lm* infection (24 hpi). These results are in accordance with a lower phosphorylation of the MAPK pathway after *Lm* infection in *Hdac6*<sup>-/-</sup> DCs, controlling the activation of the AP-1 family of TFs, which is necessary to switch-on pro-inflammatory responses [108].

In addition, the lower phosphorylation of mTORC1 pathway components in *Hdac6*<sup>-/-</sup> DCs is consistent with a lower pro-inflammatory response, as reported in trained macrophages and dendritic cells, in which a metabolic switch to glycolysis has been described [169]. These data may indicate that HDAC6 also appears to play a role in the activation of the mTOR pathway upon *Lm* infection to initiate the anti-bacterial transcriptional response to combat this pathogen.



**Figure 5.2. Proposed model of HDAC6 functions in *Lm* sensing.** Activation of membrane TLR1/2, TLR2/6 or TLR5 or endosomal TLR-9 by *Gram*-positive bacteria as *Listeria monocytogenes* initiates the TLR-signalling complex. HDAC6 binds to MyD88 independently of its deacetylases domains, suggesting a scaffold role of HDAC6 in this signalling complex, likely mediated by K63-polyUb chain recognition through HDAC6 ubiquitin binding domain (BUZ domain). Other molecules of the TLR-signalling complex, like TAB2, TAB3 and IKK proteins, have been described to be modified with K63-polyUb chains by TRAF-6 and other E3-ubiquitin ligases. NF-κB promotes the transcription of pro-inflammatory genes, whereas IRF-7 activates the transcription of type I IFNs to enhance the cell microbicidal capacity against intracellular bacteria. The images in the figure are not scaled.

Lower IFN-β secretion was detected in *Lm*-infected *Hdac6*<sup>-/-</sup> DCs from 6 to 24 hpi. Upon vacuolar escape of *Lm*, the cytosolic surveillance pathway of host cell is activated [199]. Bacterial c-di-AMP and DNA mediate the activation of STING, whereas 5'pppRNAs are sensed by RIG-I, mediating the aggregation of mitochondrial and peroxisomal MAVS and the subsequent nuclear signalling of IRF-3 and 7 [1,77]. Additionally, RIG-I deacetylation at K<sup>858</sup> and K<sup>909</sup> by HDAC6 is essential to engage MAVs for the activation of anti-viral response through IRF-3-IFN-β signalling [49,137]. The lack of RIG-I cytosolic response to *Lm* in *Hdac6*<sup>-/-</sup> DCs can partially explain the diminished pro-inflammatory production of IFN-β observed in this work.



Regarding the lower levels of RNA expression of some chemokines in *Hdac6*<sup>-/-</sup> DCs, a reduced expression of CXCL10 was also detected in HDAC6-silenced cells after HIV-1 Tat-activation [226]. However, further experiments are necessary to unveil the molecular mechanism by which HDAC6 could regulate this chemokine. In summary, our results reveal a defect in DC activation after *Lm* entry.

### 5.2.2. Pro-inflammatory cytokine secretion after stimulation with TLR agonists

The impaired anti-inflammatory response in *Hdac6*<sup>-/-</sup> DCs was also observed with other TLR-agonists such as LPS, Imiquimod, poly(I:C) and Pam3GSK4, highlighting HDAC6 as an important player in TLR signalling activation. This result remarks the possible involvement of HDAC6 in both MyD88-dependent and independent TLR-signalling pathways, because TLR-3 and non-cannonical TLR-4 pathway use TIR-domain containing adapter-inducing IFN- $\beta$  (TRIF) adaptor instead of MyD88 [234].

Broad-spectrum HDAC inhibitors such as TSA exert immunosuppressive effects in TLR-2 and TLR-4 signalling pathways [208]. Genome-wide expression profiling arrays have revealed that 60% of genes transcriptionally increased by TLR-2 or TLR-4 stimulation are inhibited in TSA-treated cells, whereas 16 % of genes are potentiated [208]. However, these observations do not provide any demonstrative evidence for a specific role of HDAC6, since other HDACs may also be involved. At this point, our results with the genetic ablation of HDAC6 unequivocally endorse a specific role for this deacetylase in TLR-mediated cytokine secretion.

### 5.2.3. TLR-signalling pathway

Because GM-CSF-derived DCs express low levels of TLR-3 and TLR-5 in the membrane, we stimulated FLT3-L-derived DCs with poly(I:C) and flagellin to measure pro-inflammatory cytokines [55,260], noting maintained failure in the inflammatory response in *Hdac6*<sup>-/-</sup> cells. Moreover, we detected impaired responses to PAMPs activation in GM-CSF-derived and FLT3L-derived *Hdac6*<sup>-/-</sup> DCs. In addition, all TLRs except for TLR-3 signal through the adaptor MyD88, and the result obtained with the TLR-3 ligand poly(I:C) was similar to that showed for the rest of TLR stimuli, thereby indicating that the TLR3-response is also affected by absence of HDAC6. In this regard, these data are in agreement with a recent study showing that acetylated retinoic-acid-inducible gene-1 (RIG-1) makes *Hdac6*<sup>-/-</sup> cells less sensitive to the presence of RNA viruses, resulting in a higher viral titre [49]. While this mechanism could explain the difference between the response of *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> FLT3L-DCs to TLR-3 stimulation, the deficient activation by other TLRs in *Hdac6*<sup>-/-</sup> DCs also requires an explanation.

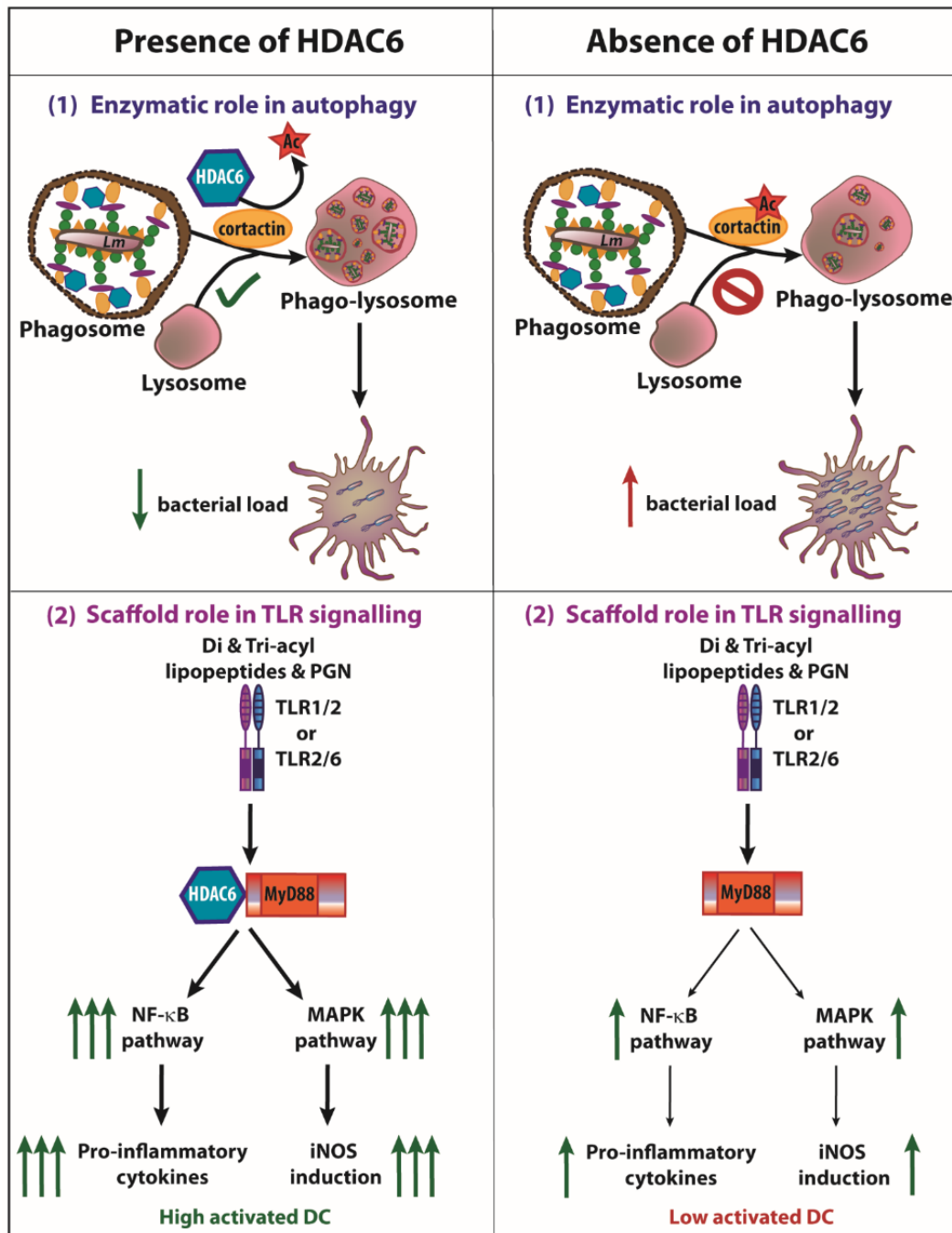
However, it has been observed that HDAC6 hampers the formation of MyD88-TRAF6 signalling complex, suppressing TLR-4 mediated activation in mouse macrophage cell line Raw 264.7 [97]. In this work, both p62 and HDAC6 are required for MyD88 aggregation and mediate the suppression of IL-6

and iNOS after TLR-ligand interaction [97]. However, these data are not in agreement with data obtained by other authors and our work [46,226,49,137].

#### 5.2.4. HDAC6-MyD88 molecular association

Since the diminished pro-inflammatory response after TLR agonists in *Hdac6*<sup>-/-</sup> DCs can be explained by RIG-I acetylation, with the exception of poly(I:C), we decided to focus our mechanistic study in the general TLR-adaptor protein MyD88. In this respect, we demonstrate the molecular association of HDAC6 with MyD88 studying the endogenous proteins and an overexpression system. Conceivably, the depletion of HDAC6 and therefore the prevention of HDAC6-MyD88 binding, could inhibit TLR-2-signalling pathway activation, which is in accordance with a lower NF-κB induction measured in *Hdac6*<sup>-/-</sup> cells after stimulation with various TLR-2 agonists. A diminished NF-κB induction in *Hdac6*<sup>-/-</sup> cells could explain a reduced initiation of the pro-inflammatory response observed in *Hdac6*<sup>-/-</sup> DCs, needed to alert the innate and adaptive immune response to *Lm*. However, NF-κB activity of *HDAC6-DD*-transfected HEK cell line after different TLR-2 stimuli (HKLM, Pam2GSK4 and Pam3GSK4) did not display any significant change compared to *HDAC6-WT*-transfected cells, supporting that enzymatic activity of HDAC6 is not involved in this signalling pathway. Interestingly, we have assessed the acetylation of MyD88 by mass spectrometry, detecting acetylation of this molecule in two residues different to the one described previously [171]. The basal MyD88 IP detected “MVVVVSDDY<sup>Ac</sup>LQS<sup>Ac</sup>K<sub>231</sub>ECDFQTK” sequence in *shHDAC6*+*MyD88*-HA and *HDAC6-DD*+*MyD88*-HA samples, and “LLELLT<sup>Ac</sup>K<sub>95</sub>LGR” sequence in *MyD88*-HA construction. The acetylated peptides detected in basal condition correspond to the MyD88 sequences “MVVVVSDDY<sup>Ac</sup>LQS<sup>Ac</sup>K<sub>231</sub>ECDFQTK” and “LLELLT<sup>Ac</sup>K<sub>95</sub>LGR”, different from the residue described by New *et al.* “L<sup>Ac</sup>K<sub>132</sub>QQQEEAEKPL”. In fact, the peptide described in New *et al* is surrounded by leucine residues, instead lysine or arginine, both produced by the trypsin digestion [171].

Our data suggest that the acetylation status of MyD88 does not appear to be regulated by HDAC6, as acetylation was detected not only in the *shHDAC6*+*MyD88*-HA sample, but also in the *HDAC6-DD*+*MyD88*-HA and *MyD88*-HA samples without any TLR stimuli. However, no changes in the acetylation marks on MyD88 were detected after HKLM incubation, highlighting the scaffold role of HDAC6 in the proper activation of TLR-signalling pathway (Figure 5.1 part 2 and Figure 5.3 part 2). Therefore, we cannot assign the defect observed in TLR-signalling in *Hdac6*<sup>-/-</sup> DCs to this post-translational modification of MyD88, as it appears to be independent of the enzymatic activity of HDAC6.



**Figure 5.3. Graphical summary of HDAC6 role during DC response to *Lm* infection.** The scheme shows the involvement of HDAC6 in two different functions of DC during *Lm* infection, the autophagy and the TLR signalling. **(1)** The fusion of phagosomes with lysosomes is dependent on cortactin and F-actin. The deacetylation of cortactin by HDAC6 allows the correct fusion, followed by an autophagic clearance of *Lm*. The absence of this enzyme delays the fusion of phagosome and lysosome, facilitating *Lm* to escape from phagosome leading to an increased bacterial load. **(2)** Di- and tri-acyl lipopeptides and peptidoglycan (PGN) of *Lm* are recognized by TLR1/2 or TLR2/6, activating the TLR pathway. HDAC6 is able to interact with the TLR-adaptor protein MyD88 which causes an enhanced down-stream signalling of the TLR pathway, increasing NF- $\kappa$ B and MAPK activation. This stronger activation (independent on HDAC6 enzymatic activity) results in higher pro-inflammatory cytokine production and iNOS induction, reinforcing the ability of the DC to combat against this intracellular pathogen. Although the absence of HDAC6 does not fully abolish DC activation, a lower induction of NF- $\kappa$ B and MAPK pathways promotes a lower activation of the anti-bacterial transcriptional program of the DCs. Note that both processes occur during *Lm* infection and the pro-inflammatory cytokines and iNOS induction can impact on the autophagic process. The images in the figure are not scaled.



It is conceivable that HDAC6 plays a scaffold role in the initial MyD88 complex in order to maintain the TLR-signalling the time necessary to achieve a proper TLR down-stream signalling. This scaffold role was previously described for HDAC6 in cell migration by Cabrero *et al* [36]. Comparison of induction of NF- $\kappa$ B with TLR-2 agonists in HEK-hTLR2 cells transfected with *HDAC6-WT*, *HDAC6-DD* and *sh-HDAC6* constructs demonstrate differences for silencing HDAC6 protein, but not for the double deacetylase mutant (DD), further supporting the scaffold role for HDAC6 in the maintenance of the TLR-signalling. However, we can not rule out that other PTMs regulate the function of MyD88 such as ubiquitination, because this molecule can be ubiquitinated at 95, 119, 231 and 262 K residues [131]. K48-linked polyubiquitinated-MyD88 seems to be degraded by Smad6-Smurf pathway after TLR-2 and TLR-4 stimulation [131]. Nevertheless, there are many types of ubiquitination and further studies are required to understand what kind of ubiquitin topology could be involved in MyD88-mediated TLR activation [114,233].

### 5.2.5. Evaluation of the HDAC6 role in the *in vivo* TLR-dependent inflammatory response

Unexpectedly, a protective effect against systemic infection to a lethal dose of *Lm* was observed in *Hdac6*<sup>-/-</sup> mice. The reduced level of the inflammatory cytokine IL-6 detected in *Hdac6*<sup>-/-</sup> mice are in accordance with its higher resistance to *Lm* infection. The defective systemic inflammatory response after *Lm* infection of *Hdac6*<sup>-/-</sup> mice may indicate an impaired TLR-response in the absence of HDAC6 and might therefore be attributed to the absence of the molecular association between MyD88 and HDAC6. In this regard, mice resistance to *Lm* infection can be mediated by sequential MyD88-independent and -dependent responses [217,66,239,218]. However, the role of TLR-2 during *Lm* infection does not appear to be clear enough [66,239]. On one hand, *Tlr-2*<sup>-/-</sup> mice display a deficit in circulating TNF- $\alpha$  and IL-12p40 production during intravenously injected *Lm* infection combined with a lower mice survival and increased bacterial burden in the liver [217,239]. Other authors have obtained similar resistance to intraperitoneal-injected *Lm* infection between *Tlr-2*<sup>-/-</sup> and *Tlr-2*<sup>+/+</sup> mice, indicating that different inoculation routes of bacteria may result in different immune outcomes [66].

Although handling and direct killing of *Lm* by activated macrophages can be mediated by TLR-2- and MyD88-independent mechanisms, the role of TLR-signalling has been found necessary for nitric oxide and cytokine production [217,239]. In fact, MyD88 not only works as TLR-adaptor protein, but also as adaptor of IL-1 and IL-18 receptors, both cytokine responses affected in *Lm*-infected *Myd88*<sup>-/-</sup> mice [66,239,6]. Although *Myd88*<sup>-/-</sup> mice are more susceptible to *Lm* infection, mice that lack caspase-1, which is responsible for the processing of immature forms of IL-1 and IL-18 into secreted forms, are only slightly more sensitive to infection than *wt* mice [66,241,217]. These data indicate that the protective immune response of MyD88 is mainly mediated through TLR-dependent signalling, rather

than IL-1 and IL-18 receptors. The recruitment of monocytes to *Lm*-infected spleens is mediated by MCP-1, which is MyD88-independent [218]. Hence, MyD88 deficiency does not impair monocyte recruitment, but prevents monocyte activation due to TNF- $\alpha$  and IL12p70 cytokine secretions are MyD88-dependent [218]. However, the protective CD8<sup>+</sup> T-cell response to *Lm* is maintained in *Myd88*<sup>-/-</sup> mice [252].

The apparently contradictory results obtained using *Tlr-2*<sup>-/-</sup> mice can be explained by different inoculation routes of *Lm*. On one hand, Torres *et al.* reported an increased susceptibility of these animals to intravenous injection of *Lm*, whereas Edelson *et al.* did not observe any difference compared to *wt* mice using intraperitoneal injection [66,239]. Moreover, *Lm* is not only recognized by TLR-2, but also by other TLRs such as TLR-9. In the case of MyD88, it does not appear to be necessary to clear bacteria in *in vitro* infections of macrophages, but this adaptor is essential for cytokine and nitric oxide production, particularly during *in vivo* infections [66,239]. Hence, MyD88 is required for early *Lm* resistance and for proper activation of innate immune responses [66].

Innate immune responses against *Lm* are essential for host survival, because the lack of various innate immunity molecules increase the susceptibility to this pathogen [242]. The fundamental role of innate immunity to combat *Lm* is highlighted in studies carried out with severe combined immunodeficient (SCID) and nude mice, which display a remarkable resistance to early time points of *Lm*-infection, but are unable to clear bacteria in the long term [14,173]. Further works have demonstrated that the IFN- $\gamma$  produced by natural killer cells is responsible for macrophage activation at early times of infection [240,34,191,209,85]. Besides, mice lacking IFN- $\gamma$  and TNF- $\alpha$  rapidly succumb to *Lm*-infection, remarking the importance of both cytokines in the control of the early infection [34,88,191,209]. However, mice that lack the receptor for type I IFNs (IFNAR1) or IRF-3 are more resistant to *Lm*-infection due to an increased apoptosis of T lymphocytes and the loss of TNF- $\alpha$ -producing cells [8,177,38]. Moreover, the lower susceptibility to *Lm* infection displayed by *Ifnar1*<sup>-/-</sup> mice was associated to a lack of cell-to-cell spreading of bacteria in *in vitro* infections of macrophages and *in vivo*, which is due to an alteration of actin-polymerization of ActA protein on the surface of *Lm* [8,230,182]. However, the existence of cellular factors that alter ActA localization of enclosing bacteria, or altered bacterial regulation mechanisms remains unknown. In this regard, HDAC6 could differently modulate host cell factors necessary for bacterial infection such as in the case of IFNAR1 absence.

Interestingly, here we detected decreased levels of the inflammatory cytokine IL-6 in *Hdac6*<sup>-/-</sup> mice serum at 72 and 96 hpi, indicating higher inflammation of *Hdac6*<sup>+/+</sup> animals in response to a lethal dose of *Lm*. Similar observations regarding lower inflammation in *Hdac6*<sup>-/-</sup> mice have been reported by other authors [46]. *Hdac6*<sup>-/-</sup> mice were more resistant to LPS injection, a model typically used to trigger a

septic shock [46]. Similarly, together the results reported in Chattopadhyay *et al.* and our *Lm* survival curves indicate that *Hdac6*<sup>-/-</sup> mice display a defect in inflammation that could protect *Hdac6*<sup>-/-</sup> mice from systemic pro-inflammatory cytokine response.

Although, the opposite results were expected based on the *in vitro* and *in vivo* data showing increased bacterial burden at early times in *Hdac6*<sup>-/-</sup> myeloid cells, a comparative of this short time with the *in vivo* response after various days of infection is hard to be interpret. Besides, HDAC6 overall effect on the inflammatory response to lethal dose of *Lm* should be taken into account when analyzing animal survival at long time points after a septic shock model. In this context, the defective response of inflammatory mediators in *Hdac6*<sup>-/-</sup> mice may be responsible for their higher survival. It has been described that MyD88 and TLR responses contribute to inflammation during infection [239]. *Tlr-2*<sup>-/-</sup> mice not only show a reduced production of TNF- $\alpha$  and IL-12, but also lower induction of iNOS, and the costimulatory molecules CD40 and CD86, necessary for the control of infection [239]. In this regard, *Lm*-infected *Hdac6*<sup>-/-</sup> GM-CSF DCs not only express lower CD86 and CD40 markers, but also a reduced nitrite production, fewer induction of iNOS and a reduced secretion of TNF- $\alpha$  and IL-12p70. However, bacterial burden can be controlled by other processes as well, e.g. macrophages directly kill *Lm* by autophagy, independently of TLR-2- and MyD88-mechanisms [66]. In this regard, the impaired autophagy observed in *Hdac6*<sup>-/-</sup> GM-CSF DCs and peritoneal macrophages at 6 hpi is not maintained during the lethal dose of *Lm*-septic shock model. This issue could be explained by a compensatory activity of HDAC5 during the absence of HDAC6 as we have observed in GM-CSF DCs after 24 hpi of *Lm*-infection. Moreover, the *in vivo* quantification of bacterial burden in target organs from 12 to 48 hpi, did not display any significant results between *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> mice, indicating that compensatory mechanisms are playing at these times or perhaps, the inflammatory cytokine storm is impairing the bacterial clearance at 72 hpi. Upon *Lm* infection, host cell Sirt-2 is shuttled to the nucleus to deacetylate histone 3, changing the chromatin packaging and the access of TFs. This step seems to be required for effective *Lm* infection because Sirt-2-deficient mice display decreased bacterial burden in target organs [67]. The molecular association between Sirt-2 and HDAC6 has been previously reported. Although HDAC6 is able to bind to both phosphorylated and non-phosphorylated forms of Sirt-2, the ability of Sirt-2 to bind tubulin can only be mediated when it forms a complex with HDAC6 [164]. HDAC6-Sirt-2 interaction could mediate also a role in the nucleus by deacetylating histone 3, and the absence of HDAC6 may alter *Lm* adaptation in the host cell. This could explain why *Hdac6*<sup>-/-</sup> mice are protected from *Lm* infection as well as *Sirt-2*<sup>-/-</sup> mice.

The results obtained with *in vivo* long-term infection experiments may seem contradictory compared to *in vitro* assays and to short-term *in vivo* ones. Hence, further studies will be needed to uncover the consequences of HDAC6 deficiency in other mouse cell populations, in other kind of

bacterial inoculation, like intraperitoneal or oral infection models, or in other contexts of bacterial infection, such as *Gram*-negative bacteria.

Taking together, our data support a dual role for HDAC6 in the regulation of innate immunity against intracellular bacteria. An increased bacterial load in different *Hdac6*<sup>-/-</sup> myeloid cells can be explained by the autophagy mechanism, where a permanently acetylated cortactin may impair the phagosome-lysosome fusion, necessary for the clearance of this pathogen. Our experiments also show the importance of HDAC6 in DC activation, uncovering a novel mechanism of HDAC6 action mediated by the appropriate signalling via the TLR pathway, due to the association of HDAC6 with the TLR-adaptor protein MyD88. This molecular association seems to be required for a response to TLR stimuli to initiate the inflammatory response of an activated DC. Taken together, both HDAC6 functions described in this work, reinforce the importance of this molecule to combat the intracellular bacteria *Lm* by autophagy and to completely activate the inflammatory response after TLR activation. Overall, this work identifies for the first time part of the TLR regulatory machinery of the proinflammatory response after *Lm* infection. Moreover, the results obtained demonstrate the importance of HDAC6 in the control of intracellular bacterial infection by innate immune system.





# Conclusions





## 6. CONCLUSIONS

The findings presented herein support the following conclusions:

1. HDAC6 protein expression is increased during *Lm* infection in DCs.
2. *Hdac6*<sup>-/-</sup> myeloid cells display defective clearance of intracellular bacteria due to an impaired autophagy.
3. *Hdac6*<sup>-/-</sup> DCs display higher titres of *Lm* due to delayed fusion of autophagosome with lysosome, likely mediated by the acetylation state of cortactin.
4. *Hdac6*<sup>-/-</sup> DCs show impaired pro-inflammatory cytokine production in response to *Lm* infection and TLR agonist stimulation.
5. *Hdac6*<sup>-/-</sup> DCs exhibit weaker iNOS microbicidal activity after *Lm* infection and HKLM stimulation.
6. *Hdac6*<sup>-/-</sup> DCs show lower induction of MAPK and mTOR signalling pathways after *Lm* infection and TLR activation, indicating a defective anti-bacterial response.
7. There is a molecular association between HDAC6 and MyD88 responsible of a proper inflammatory response after TLR stimulation, which is independent of HDAC6 enzymatic activity.
8. The impaired TLR-signalling pathway observed in the absence of HDAC6 can explain the *Hdac6*<sup>-/-</sup> mice protection against septic shock model based in lethal dose-*Lm* infection.



Conclusiones



## 7. CONCLUSIONES

Los resultados presentados en esta tesis doctoral son respaldados con las siguientes conclusiones:

1. La expresión de la proteína HDAC6 se induce durante la infección de DCs con *Lm*.
2. Las células mieloides de ratones *Hdac6*<sup>-/-</sup> muestran un defecto en la eliminación de bacterias intracelulares debido a un defecto en la autofagia.
3. Las DCs de ratones *Hdac6*<sup>-/-</sup> contienen una mayor carga bacteriana debido a un retraso en la fusión del autophagosoma con el lisosoma, probablemente mediada por la acetilación de la cortactina.
4. En las DCs de ratones *Hdac6*<sup>-/-</sup> se detecta una producción de citoquinas proinflamatorias disminuida en respuesta a la infección por *Lm* y a la estimulación con ligandos de TLRs.
5. En las DCs de ratones *Hdac6*<sup>-/-</sup> DCs se observa una menor inducción de la actividad microbicida de la enzima iNOS tras la infección con *Lm* y la estimulación con HKLM.
6. Las DCs de ratones *Hdac6*<sup>-/-</sup> DCs tienen una menor inducción de las rutas de señalización de MAPK y mTOR por la infección de *Lm* y la activación de los TLR, lo que indica que la respuesta anti-bacteriana está afectada.
7. La asociación molecular existente entre HDAC6 y MyD88 es responsable de la correcta respuesta inflamatoria después de la estimulación de los TLRs, que parece ser independiente de la actividad enzimática de HDAC6.
8. La ruta de señalización de los TLRs afectada que se observa en la ausencia de la proteína HDAC6, podría explicar la protección de los ratones *Hdac6*<sup>-/-</sup> frente al modelo de shock séptico usando una dosis letal infectiva de *Lm*.



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# Annexes





## 10.ANNEXES

### 10.1. Publications related to this thesis

1. **HDAC6 controls innate immune and autophagy responses to the intracellular bacteria *Listeria monocytogenes*.** Moreno-Gonzalo O, Ramírez-Huesca M, Blas-Rus N, Cibrián D, Saiz M.L, Jorge I, Camafeita E, Vázquez J, Sánchez-Madrid F. *PLOS Pathogens*. 2017. doi: 10.1371/journal.ppat.1006799. PMID:29281743.
2. **Deacetylase at crossroads of infection and innate immunity.** Moreno-Gonzalo O, Mayor F Jr, Sánchez-Madrid F. *Trends in Immunology*. 2018. (submitted).

### 10.2. Other Publications

1. **Tetraspanin CD9 limits mucosal healing in experimental colitis.** Saiz ML, Cibrián C, Ramírez-Huesca M, Torralba D, Moreno-Gonzalo O, Sánchez-Madrid F. *Frontiers in Immunology*. 2017. <https://doi.org/10.3389/fimmu.2017.01854>
2. **Post-Translational add-ons mark the path in exosomal protein sorting.** Moreno-Gonzalo O, Fernandez-Delgado I, Sánchez-Madrid F. *Cellular and Molecular Life Science*. 2017. doi: 10.1007/s00018-017-2690-y. PMID:29080091.
3. **ISGylation controls exosome secretion by promoting lysosomal degradation of MVB proteins.** Villarroja-Beltri C, Baixauli F, Mittelbrunn M, Fernández-Delgado I, Torralba D, Moreno-Gonzalo O, Baldanta S, Enrich C, Guerra S, Sánchez-Madrid F. *Nature Communications* (2016) 7; 13588. doi: 10.1038/ncomms13588. PMID: 27882925.
4. **CD69 controls the uptake of L-tryptophan through LAT1-CD98 and AhR-dependent secretion of IL-22 in psoriasis.** Cibrián D, Saiz ML, de la Fuente H, Sánchez-Díaz R, Moreno-Gonzalo O, Jorge I, Ferrarini A, Vázquez J, Punzón C, Fresno M, Vicente-Manzanares M, Daudén E, Fernández-Salguero PM, Martín P, Sánchez-Madrid F. *Nature Immunology*. 2016 Aug;17(8):985-96. doi: 10.1038/ni.3504. Epub 2016 Jul 4. PMID: 27376471.
5. **HDAC6 regulates the dynamics of lytic granules in cytotoxic T lymphocytes.** Núñez-Andrade N, Iborra S, Trullo A, Moreno-Gonzalo O, Calvo E, Catalán E, Menasche G, Sancho D, Vázquez J, Yao TP, Martín-Cófreces NB, Sánchez-Madrid F. *Journal of Cell Science*. 2016 Apr 1;129(7):1305-11. doi: 10.1242/jcs.180885. Epub 2016 Feb 11. PMID: 26869226.
6. **Post-translational modifications of exosomal proteins.** Moreno-Gonzalo O, Villarroja-Beltri C, Sánchez-Madrid F. *Frontiers in Immunology*. 2014 Aug 11;5:383. doi: 10.3389/fimmu.2014.00383. eCollection 2014. Review. PMID: 25157254.
7. **The PDZ-adaptor protein syntenin-1 regulates HIV-1 entry.** Gordón-Alonso M, Rocha-Perugini V, Álvarez S, Moreno-Gonzalo O, Ursa A, López-Martín S, Izquierdo-Useros N, Martínez-Picado J, Muñoz-Fernández MÁ, Yáñez-Mó M, Sánchez-Madrid F. *Molecular Biology of the Cell*. 2012 Jun;23(12):2253-63. doi: 10.1091/mbc.E11-12-1003. Epub 2012 Apr 25. PMID: 22535526.



## Selected Articles



## **12. SELECTED ARTICLES**

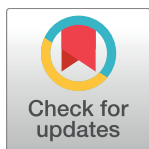
RESEARCH ARTICLE

# HDAC6 controls innate immune and autophagy responses to TLR-mediated signalling by the intracellular bacteria *Listeria monocytogenes*

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## Abstract

Recent evidence on HDAC6 function underlines its role as a key protein in the innate immune response to viral infection. However, whether HDAC6 regulates innate immunity during bacterial infection remains unexplored. To assess the role of HDAC6 in the regulation of defence mechanisms against intracellular bacteria, we used the *Listeria monocytogenes* (*Lm*) infection model. Our data show that *Hdac6*<sup>-/-</sup> bone marrow-derived dendritic cells (BMDCs) have a higher bacterial load than *Hdac6*<sup>+/+</sup> cells, correlating with weaker induction of IFN-related genes, pro-inflammatory cytokines and nitrite production after bacterial infection. *Hdac6*<sup>-/-</sup> BMDCs have a weakened phosphorylation of MAPK signalling in response to *Lm* infection, suggesting altered Toll-like receptor signalling (TLR). Compared with *Hdac6*<sup>+/+</sup> counterparts, *Hdac6*<sup>-/-</sup> GM-CSF-derived and FLT3L-derived dendritic cells show weaker pro-inflammatory cytokine secretion in response to various TLR agonists. Moreover, HDAC6 associates with the TLR-adaptor molecule Myeloid differentiation primary response gene 88 (*MyD88*), and the absence of HDAC6 seems to diminish the NF-κB induction after TLR stimuli. *Hdac6*<sup>-/-</sup> mice display low serum levels of inflammatory cytokine IL-6 and correspondingly an increased survival to a systemic infection with *Lm*. The impaired bacterial clearance in the absence of HDAC6 appears to be caused by a defect in autophagy. Hence, *Hdac6*<sup>-/-</sup> BMDCs accumulate higher levels of the autophagy marker p62 and show defective phagosome-lysosome fusion. These data underline the important function of HDAC6 in dendritic cells not only in bacterial autophagy, but also in the proper activation of TLR signalling. These results thus demonstrate an important regulatory role for HDAC6 in the innate immune response to intracellular bacterial infection.

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**Competing interests:** The authors have declared that no competing interests exist.

## Author summary

*Listeria monocytogenes* (*Lm*) is a food-borne intracellular bacterium that causes listeriosis to 1.600 people each year, being responsible of approximately 260 deaths. This pathogen mostly affects immunocompromised individuals and pregnant women. It is particularly dangerous for the later due to its ability to pass across the placenta and the blood-brain barrier. *Lm* is extensively used as a *Gram* positive infection model in the laboratory to study innate and adaptive immune responses. HDAC6 is an important regulatory enzyme of the tubulin and actin cytoskeletons. Its inhibition or deficiency quells the immune response against different virus infections. Previous work has shown its involvement in the regulation of viral RNA-sensing activity and in interferon signalling. In this study, we report that HDAC6 is an essential component of the innate immune response to fight against intracellular bacterial infections. Genetic ablation of HDAC6 impairs activation of the pertinent Toll-like receptor pathway to induce the pro-inflammatory transcriptional program of the cell. Moreover, this enzyme controls cytoskeletal proteins that mediate the fusion of phagosome-contained bacteria with the lysosome during pathogen degradation.

## Introduction

Histone deacetylase 6 (HDAC6) is a cytoplasmic deacetylase involved in the regulation of several biological processes, including migration, transport, angiogenesis, and tumour progression [1–5]. This enzyme is able to deacetylate  $\alpha$ -tubulin and cortactin, regulating not only the microtubule cytoskeleton, but also actin [6, 7]. Both cytoskeletal interactions underline a crucial role of HDAC6 in many cellular functions such as phagosome-lysosome fusion, cargo transport through microtubules, and cell motility [8–10]. The role of HDAC6 has also been described in two of the main cellular degradation mechanisms: autophagy, through interaction with the autophagy marker p62; and the proteasome, mediated by deacetylation of HSP90 and its intersection with the ubiquitin-proteasome system (UPS) [11–15]. In addition, HDAC6 is involved in the transport of damaged mitochondria (mitophagy) and misfolded proteins (aggrephagy) to lysosomes and the proteasome for degradation [16–18]. The absence of HDAC6 impairs the deacetylation of mitofusin 1, preventing the mitochondrial fusion induced by glucose deprivation and causing excessive ROS production that provokes oxidative damage [19].

HDAC6 regulates the replication of human immunodeficiency virus (HIV) by deacetylating Tat and thus inhibiting viral transactivation [20, 21]. HDAC6 also participates in Sendai virus infection through the deacetylation of  $\beta$ -catenin, which acts as a co-activator of IRF3-mediated transcription [22]. During infection with Influenza Virus A (IVA), HDAC6 appears to play a dual role. IVA capsids mimic misfolded-protein aggregates to take advantage of the host cell aggresome pathway, thereby achieving disassembly and successful viral uncoating [23]. On the other hand, HDAC6-mediated microtubule deacetylation impairs the IVA cycle, preventing trafficking of viral components to the viral assembly site in the host plasma membrane and the spread of infection to surrounding cells [24]. The role of HDAC6 in the adaptive CD4 + T-cell response has been studied in several autoimmune and inflammatory situations such as colitis and cardiac allograft rejection; however, little is known about its role in innate immunity and bacterial diseases [25, 26].

*Listeria monocytogenes* (*Lm*) is a *Gram*-positive bacteria that causes severe infection in immunocompromised individuals and is able to cross the blood-brain barrier and the placenta [27]. *Lm* is widely used as a model of innate and adaptive immune responses to intracellular

bacterial infection [27–29]. From the first hours of infection, professional phagocytic cells trap bacteria in the blood and target organs, exerting a degree of control on bacterial growth [28]. After internalization by phagocytic cells, *Lm* is eliminated by fusion of the phagosome with lysosomes; however, some bacteria escape the phagosome into the cytoplasm through the action of listeriolysin O (LLO). In the cytoplasm, *Lm* replicates and is able to infect neighbouring cells [30–32]. Interestingly, phagosome-contained bacteria are also eliminated by the action of reactive oxygen species (ROS) and nitric oxide (NO), produced by NADPH oxidase 2 (NOX2) and inducible NO synthase (iNOS), respectively [33]. Moreover, *Lm* bacteria contain an ARP2/3-mimicking protein that enables their propulsion to neighbouring cells through the directional assembly of actin filaments (actin rockets) [34]. *Lm* can spread from cell to cell without exiting the intracellular compartment by a process called paracytophagy, which evades immune detection. However, the host cell is able to develop a specific CD8<sup>+</sup>T cell response to cytosolic *Lm*, which is crucial for the control of infection [35–38].

Early control of *Listeria* burden largely depends on the innate immune response occurring in the spleen, which relies on two main cell populations of dendritic cells (DCs). On one hand, a subset of monocyte-derived DCs namely TNF/iNOS-producing DCs (Tip-DCs) has the ability to produce TNF $\alpha$  and NO [39]. The other splenic DC subset is CD8 $\alpha$ <sup>+</sup> conventional DCs (cDCs), and it is responsible for the final resolution of infection against *Listeria* through the antigen presentation of bacterial-derived antigens to specific CD8<sup>+</sup>T cells to induce cytotoxicity [40, 41]. The response of dendritic cells (DC) to live *Lm* is mediated by toll-like receptors (TLRs), nucleotide-binding oligomerization domain (NODs)-like receptors (NLRs), and other cytosolic receptors and involves two signalling pathways: TLR-dependent and independent signalling. TLR-dependent signalling, triggered by sensing of cell-surface and endo-phagosomal bacteria, mediating the activation of a MyD88-dependent response; and the cytosolic pathway, triggered by bacterial DNA after the escape of *Lm* into the cytosol, is responsible for the activation of sensor stimulator of interferon (IFN) genes (STING). STING activation leads to IFN regulatory factor (IRF)3-dependent production of IFN- $\beta$  and activation of downstream signals that control the transcription of IFN target genes essential for antiviral and antibacterial responses [42, 43].

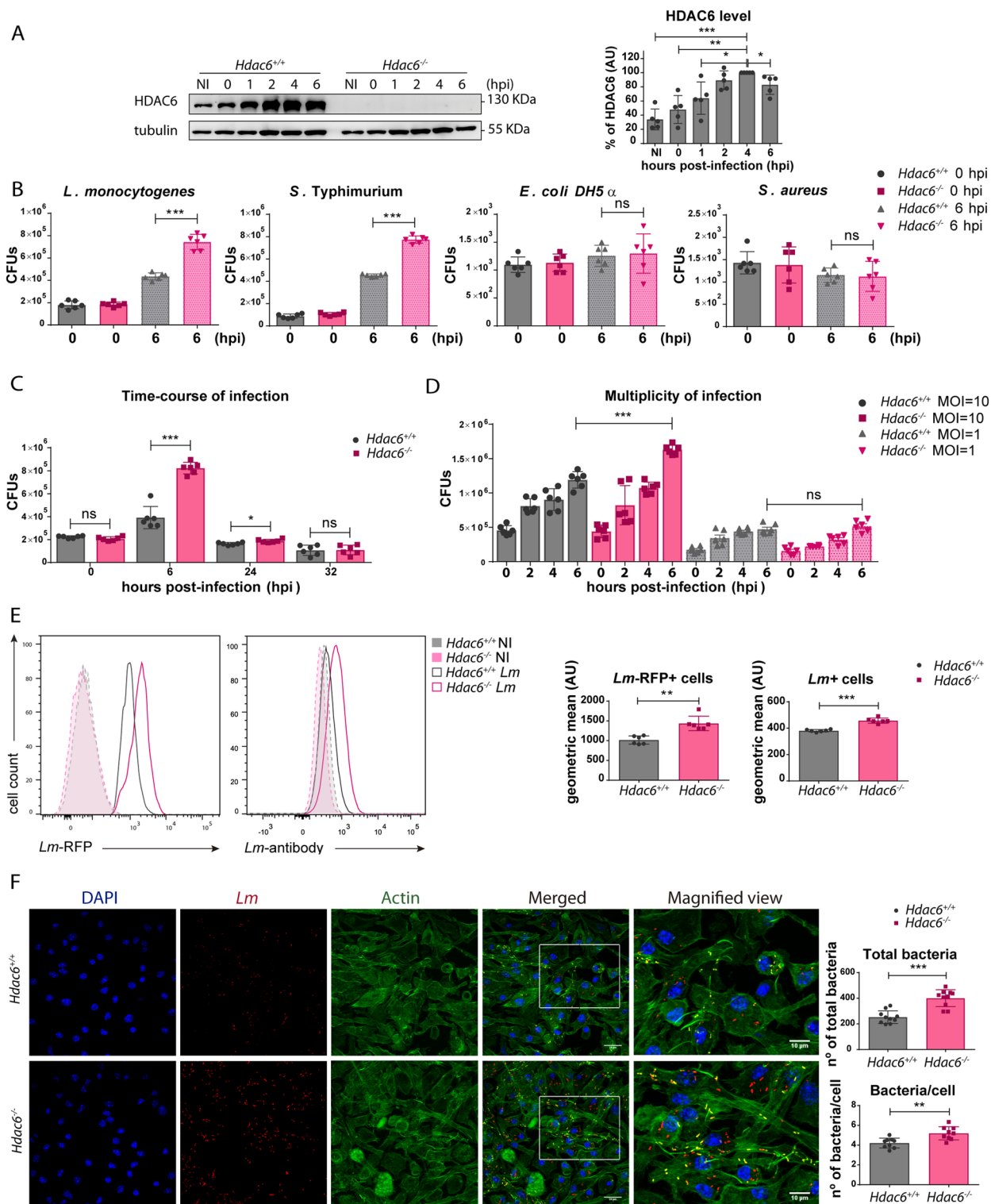
To determine the role of HDAC6 in the innate response to bacterial infection, we explored the impact of HDAC6 deficiency on the response of myeloid cells to *Lm*. Our results reveal that *Hdac6*<sup>-/-</sup> BMDCs are less efficient than *Hdac6*<sup>+/+</sup> at clearing *Lm*. This is due to defective maturation of phagosome-contained bacteria. Moreover, *Hdac6*<sup>-/-</sup> DCs display lower activation after *Lm* infection and TLR stimuli. These data support the view that HDAC6 positively regulates innate defence mechanisms against *Lm* and that its absence weakens the pro-inflammatory response.

## Results

### Deficient intracellular bacteria clearance in *Hdac6*<sup>-/-</sup> BMDCs

To assess the possible role of HDAC6 in innate immune responses during bacterial pathogenesis, we performed a time-course infection with *Lm* in granulocyte and monocyte colony-stimulating factor (GM-CSF)-derived BMDCs from *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> mice. Increasing levels of HDAC6 expression were detected in the *Hdac6*<sup>+/+</sup> DCs as the infection progressed (Fig 1A). However, BMDC differentiation was not noticeably affected in the absence of HDAC6 (S1 Fig part A). Next, *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> BMDCs were infected for different times with *Gram*-negative bacteria (*Salmonella* Typhimurium and *Escherichia coli* DH5 $\alpha$ ) and *Gram*-positive bacteria (*Listeria monocytogenes* and *Staphylococcus aureus*) at a multiplicity of infection (MOI) of 10, with colony-formed units (CFUs) corresponding to intracellular live bacteria. Bacterial





**Fig 1. Deficient intracellular bacteria clearance in *Hdac6*<sup>-/-</sup> BMDCs.** A) Western blot analysis of HDAC6 in a time-course of infection of BMDCs with *Lm*. Tubulin was used as a loading control. HDAC6 levels were quantified in five independent experiments. \*\*\*p<0.001, \*\*p<0.01, \*p<0.05. B) CFUs obtained at 0 and 6 hpi from BMDCs infected with *L. monocytogenes*, *S. Typhimurium*, *E. coli DH5α*, and *S. aureus* at a MOI of 10. Data from 0 hpi are shown as a bacteria entry control. \*\*\*p<0.001, ns>0.05 non-significant; n = 6. C) CFUs of *Lm*-infected BMDCs obtained at 0, 6, 24 and 32 hpi with a MOI = 10. \*\*\*p<0.001, \*p<0.05, ns>0.05 non-significant; n = 6. D) CFUs of *Lm*-infected BMDCs obtained at 0, 2, 4 and 6 hpi with a MOI = 10 and 1. \*\*\*p<0.001, \*p<0.05, ns>0.05 non-significant; n = 6. E) BMDCs were infected with *Lm* or *Lm*-RFP for 6 h and the bacterial signal

was determined by flow cytometry. The panel shows representative histograms and the geometric mean of the *Lm* signal. \*\*\*  $p \leq 0.001$ , \*\*  $p \leq 0.01$ ;  $n = 6$ . F) Confocal microscopy determination of bacterial load at 6 hpi. *Left panel*: Maximum intensity z-projections of confocal microscopy images of *Lm*-infected *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> BMDCs at 6 hpi. The panel shows DAPI (blue), *Lm* (red),  $\beta$ -actin (green), merged views of three channels, and magnified views of the boxed areas from the merged view. Yellow indicates *Lm* and  $\beta$ -actin co-localization. Scale bars 20  $\mu$ m (main panels) and 10  $\mu$ m magnified views). *Right panel*: ImarisCell Module analysis of the number of cells and the number of bacteria per cell in all pictures (10 pictures per genotype). Statistical analysis of Imaris quantification of total bacteria and bacteria per cell in *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> BMDCs. \*\*\*  $p \leq 0.001$ , \*\*  $p \leq 0.01$ ;  $n = 10$ .

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entry was similar in *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> DCs at 0 h post-infection (hpi), while bacterial proliferation, measured at 6 hpi, was significantly higher in *Hdac6*<sup>-/-</sup> BMDCs for both types of intracellular pathogens, *Lm* and *S. Typhimurium* (Fig 1B). This was not due to differences in cell viability at 6 hpi (S1 Fig part B). In contrast, no significant difference was observed in the proliferation of the non-intracellular pathogens *S. aureus* and *E. coli*, indicating that HDAC6 is an important component of cellular mechanisms for the clearance of intracellular pathogens (Fig 1B).

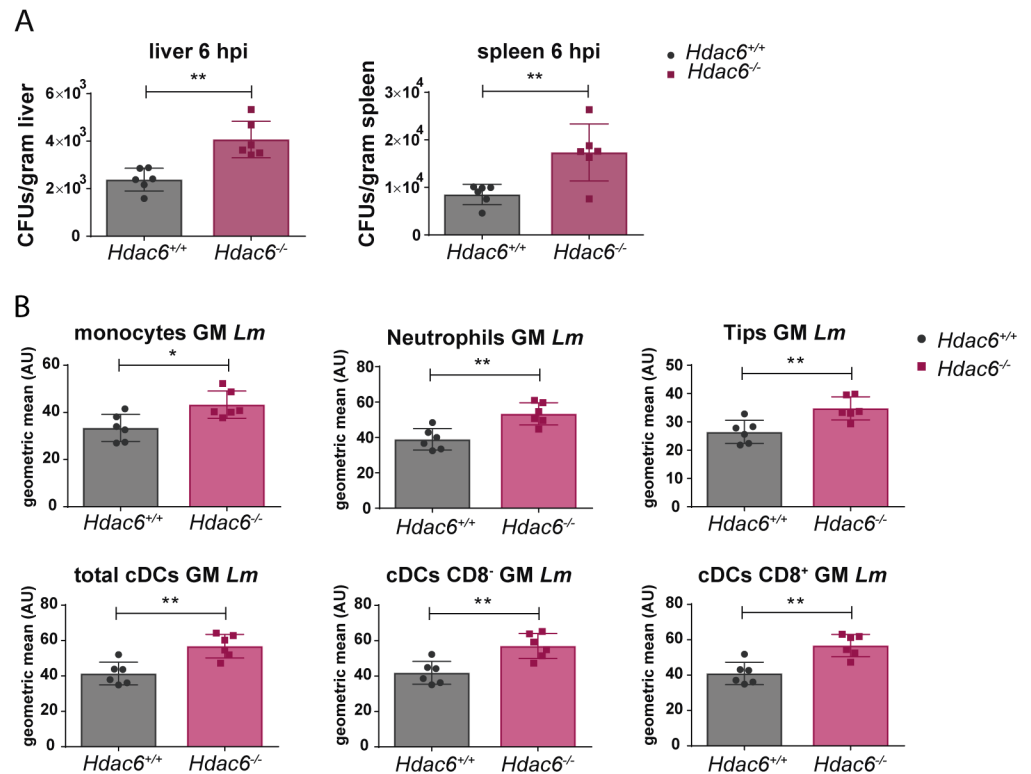
Time-course analysis showed that differences between *Lm* infection in *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> BMDCs CFUs peaked at 6 hpi and were sustained until 24 hpi (Fig 1C). This effect was clearly observed at a MOI of 10, which did not affect cell viability (Fig 1D and S1 Fig part B). A similar pattern was observed with macrophage colony-stimulating factor (M-CSF)-derived macrophages, demonstrating the lineage independence of the role of HDAC6 in bacterial clearance (S1 Fig part C). Although the difference between *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> cells was observed in both macrophages and DCs, the clearance capacity of macrophages was ten-fold higher than that of DCs at 6 hpi (S1 Fig part C).

Bacterial load was also determined by flow cytometry using two strategies: a specific antibody against *Lm*, and RFP-expressing bacteria. Both approaches showed that *Hdac6*-deficient DCs contained more bacteria at 6 hpi (Fig 1E). Higher numbers of bacteria in *Hdac6*<sup>-/-</sup> BMDCs were also detected by confocal fluorescence microscopy at 6 hpi (Fig 1F). Some bacteria co-localized with filamentous actin, showing clear actin rockets (Fig 1F). Image quantification confirmed that *Hdac6*<sup>-/-</sup> BMDCs contained more bacteria per cell and more total bacteria, remarking a higher percentage of cells hosting a large number of bacteria in *Hdac6*<sup>-/-</sup> cells (see distribution of bacteria per cell, 6–7) (S1 Fig part D). ImarisCell Module view of Fig 1F images showed the number of bacteria per cell using actin transparency to easily visualize individual bacteria (S1 Fig part E).

To ascertain whether *Hdac6*<sup>-/-</sup> cells display higher bacterial burden than *Hdac6*<sup>+/+</sup> cells *in vivo*, *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> mice were intravenously injected with *Lm* and total CFUs per gram of liver and spleen were determined at 6 hpi. In agreement with the higher numbers of *Lm* observed in GM-CSF-DCs and M-CSF-Macrophages, we observed increased bacterial titres in spleen and liver cell suspensions (Fig 2A). Next, to determine the specific cell populations underlying this phenotype, a multicolour gating strategy was used to identify the myeloid cell compartment, including monocytes, neutrophils, TIPS DCs, total cDCs, cCDs CD8<sup>-</sup> and cCDs CD8<sup>+</sup> (S2 Fig part A). Higher numbers of *Lm* were observed in different myeloid cells at 6 hpi (Fig 2B and S2 Fig part B). These data highlight the impairment of *Hdac6*<sup>-/-</sup> myeloid cells to clear intracellular *Lm*.

### Impaired bacterial clearance in *Hdac6*<sup>-/-</sup> BMDCs is caused by a defect in autophagy

To test the involvement of autophagy in the mechanism by which HDAC6 regulates *Lm* infection, we treated DCs with 3-methyladenine (3-MA), an inhibitor of autophagosome formation. Treatment with 3-MA increased bacterial load in *Hdac6*<sup>+/+</sup> BMDCs at 6 hpi, while having no



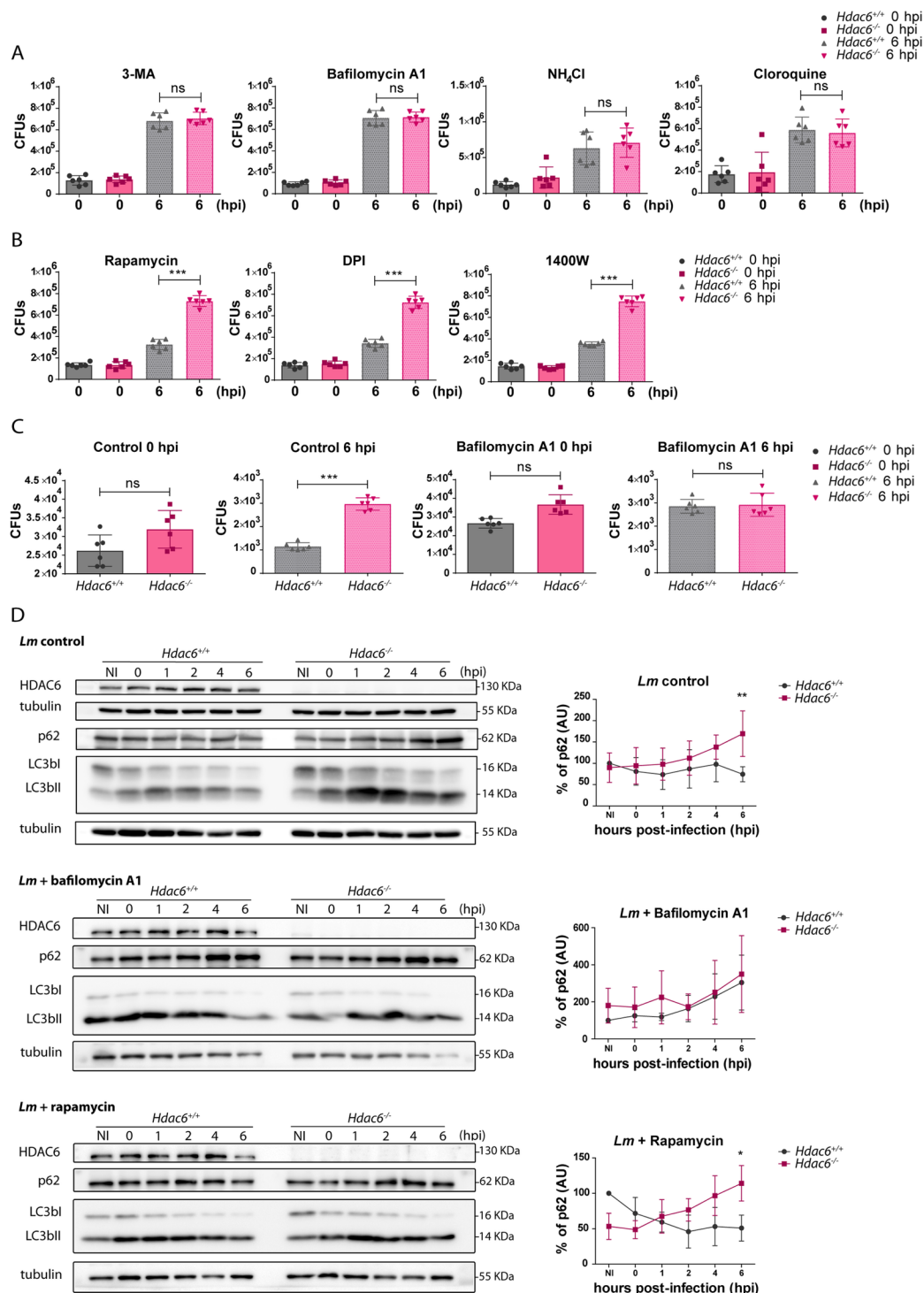
**Fig 2. Deficient intracellular bacteria clearance in *Hdac6*<sup>-/-</sup> splenic myeloid populations.** A) Quantification of bacterial load in target organs (spleen and liver) at 6 hpi in *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> mice injected with a lethal dose of *Lm*. Bacterial load is expressed by CFUs per gram of liver (left graph) and per gram of spleen (right graph). \*\**p* ≤ 0.01, *n* = 6. B) The charts show geometric means of *Lm* of different splenic populations (monocytes, neutrophils, Tips DCs, total cDCs, cDCs CD8<sup>-</sup> and cDCs CD8<sup>+</sup>) gated in the live CD3<sup>+</sup>CD19<sup>-</sup>DX5<sup>-</sup> population of *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> mice injected with a lethal dose of *Lm* at 6 hpi. \*\**p* ≤ 0.01; *n* = 6.

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effect on *Hdac6*<sup>-/-</sup> BMDCs (Fig 3A), suggesting autophagy as the bacterial clearance mechanism impaired in *Hdac6*-deficient DCs. A similar result was observed upon treatment of BMDCs with bafilomycin A1, an inhibitor of vacuolar proton pump that indirectly inhibits phagosome-lysosome fusion, and with the lysosome acidification inhibitors chloroquine and NH<sub>4</sub>Cl (Fig 3A). In contrast, increasing autophagy flux with rapamycin did not restore the impaired autophagy in *Hdac6*<sup>-/-</sup> BMDCs (Fig 3B). No significant effects were observed with control vehicles (S3 Fig part A). To explore other possible mechanisms, we treated BMDCs with inhibitors of NADPH oxidase (DPI) and iNOS (1400W). These treatments did not alter the difference in CFU number at 6 hpi between treated and non-treated *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> BMDCs, indicating that the activity of either enzyme is not accounting for the existing phenotype (Fig 3B).

The defective autophagy phenotype of *Hdac6*<sup>-/-</sup> BMDCs was not due to transcriptional alterations to autophagy or lysosome components, since *Lm*-infected *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> BMDCs showed no mRNA expression differences at 6 hpi in the autophagy components LC3A and B, p62, ATG2, 5, 7 and 12, and Beclin-1 or in the lysosome components LAMP-1 and 2 (S3 Fig part B).

To determine whether these findings can be extended to other phagocytic cells, we carried out CFU assays with macrophages obtained from *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> mice four days after intraperitoneal thioglycollate injection. Higher bacterial load was observed only in *Hdac6*<sup>-/-</sup>



**Fig 3. Impaired bacterial clearance in *Hdac6*<sup>-/-</sup> BMDCs is caused by a defect in autophagy.** A) Total CFUs in *Lm*-infected BMDCs treated with inhibitors. CFUs were detected at entry (0 hpi) and 6 hpi (bacterial proliferation) using the autophagy inhibitors (3-MA and bafilomycin A1 and the lysosome acidification inhibitors ( $\text{NH}_4\text{Cl}$  and cloroquine), ns>0.05 non-significant; n = 6. B) Total CFUs at 0 and 6 hpi in *Lm*-infected BMDCs treated with the autophagy activator (rapamycin), the NADPH oxidase inhibitor (DPI) and the iNOS inhibitor (1400W). \*\*\* $p \leq 0.001$ ; n = 6. C) Total CFUs at 0 and 6 hpi in *Lm*-infected thioglycollate-elicited macrophages treated with or without bafilomycin A1. \*\*\* $p \leq 0.001$ , ns>0.05 non-significant; n = 6. D) Western-blot analysis of autophagy markers over the time-course of *Lm* infection in *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> BMDCs. Left panels: Levels were detected of p62, LC3bI and II and HDAC6 in control cells and cells treated

with bafilomycin A1 and rapamycin. Tubulin was used as a loading control. HDAC6 was as a genotype check of *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> BMDCs and to monitor HDAC6 induction during infection. *Right panels*: Accompanying charts show quantification of the p62 percentage of control, bafilomycin A1 and rapamycin western blots. \*\*  $p \leq 0.01$ , \*  $p \leq 0.05$ , ns > 0.05 non-significant; n = 5.

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macrophages at 6 hpi, and this difference was abrogated by treatment with bafilomycin A1 (Fig 3C). These data indicate that the phenotype observed in BMDCs is also extendable in other *Hdac6*-deficient phagocytic cells such as peritoneal macrophages, indicating a widespread defect in intracellular killing ability due to lack of HDAC6. Moreover, the killing ability shown by peritoneal macrophages is similar to that of M-CSF-derived macrophages and higher than GM-CSF-derived DCs (Fig 3C compared with S1 Fig part C).

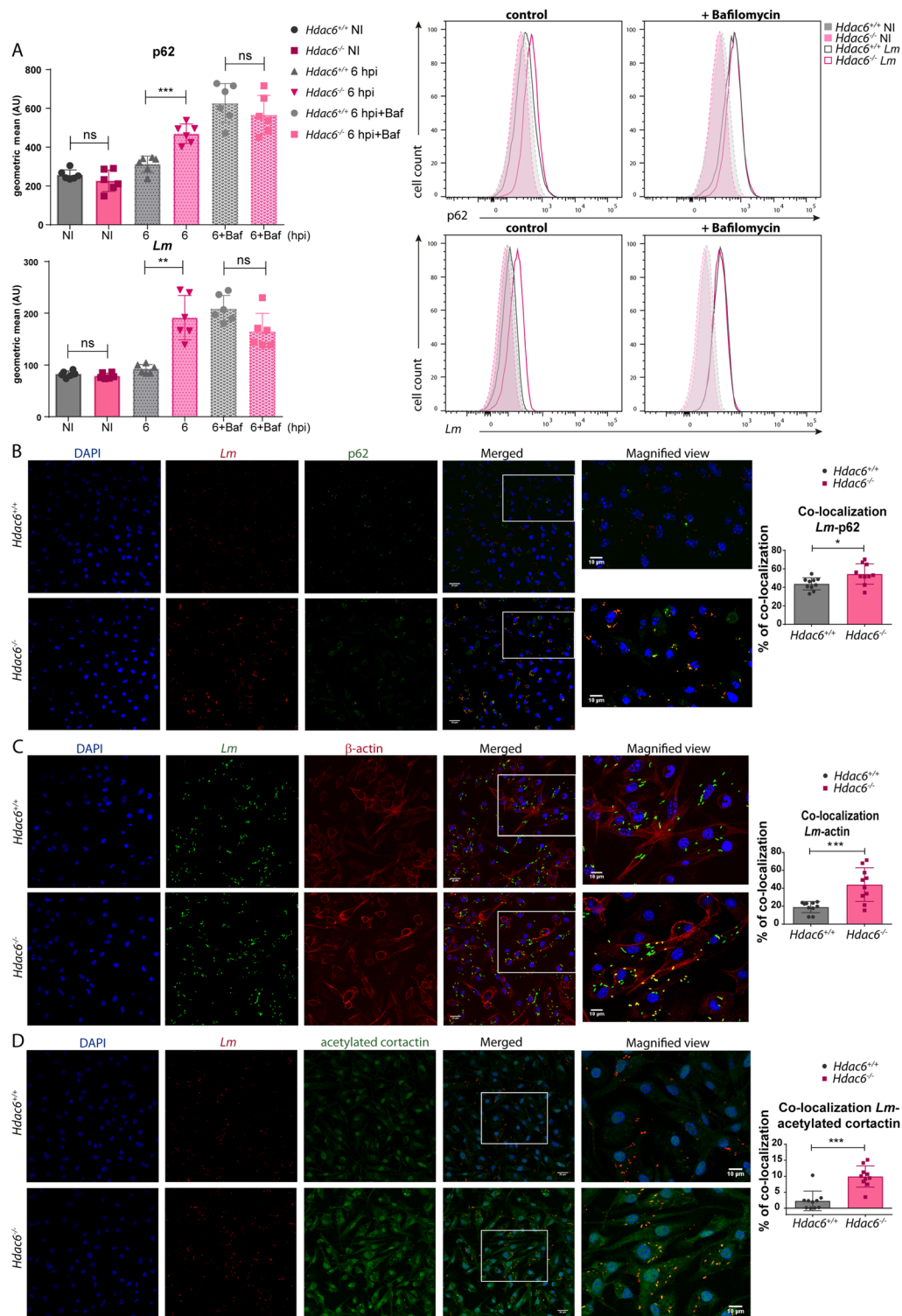
To gain further insight into the autophagy mechanism affected by HDAC6, we monitored the autophagosome markers p62 and LC3bI and II in *Lm*-infected BMDCs. *Hdac6*<sup>-/-</sup> BMDCs showed a 2-fold higher accumulation of p62 than *Hdac6*<sup>+/+</sup> cells at 6 hpi and increased LC3bII level in *Hdac6*<sup>+/+</sup> cells from 1 to 6 hpi (Fig 3D). However, differences in p62 and LC3b levels were not noticed at early times of *Lm* infection of *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> BMDCs, indicating that the induction of autophagy is not affected in the absence of HDAC6 (Fig 3D). The treatment with bafilomycin A1 enhances the accumulation of p62 during the infection at the same level in both genotypes, abrogating the deficiency in autophagy observed in *Hdac6*<sup>+/+</sup> BMDCs (Fig 3D). Although rapamycin also increased p62 accumulation at early times in *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> BMDCs, only *Hdac6*<sup>+/+</sup> cells are able to diminish p62 at 6 hpi (Fig 3D). This treatment confirmed the results obtained in the CFUs functional assays with this inhibitor (Fig 3D compared with Fig 3B). The similarity of the autophagy defect detected in *Hdac6*<sup>-/-</sup> BMDCs in control condition to that in rapamycin-treated *Hdac6*<sup>-/-</sup> cells, suggests an impairment in phagocytic vesicle fusion with the lysosome.

### *Hdac6*<sup>-/-</sup> BMDCs accumulate p62

In order to further understand the defective autophagy of *Hdac6*<sup>-/-</sup> BMDCs, the accumulation of p62 was studied in more detail. Flow cytometry at 6 hpi revealed significantly higher p62 content in *Hdac6*<sup>-/-</sup> BMDCs, indicating accumulation of this phagosome marker due to defective fusion of this organelle with the lysosome (Fig 4A). Bafilomycin A1 treatment completely abrogated this difference, suggesting that *Hdac6*<sup>-/-</sup> BMDCs displayed an impairment in the final step of autophagy (Fig 4A). More signal of *Lm* is displayed in *Hdac6*<sup>-/-</sup> DCs (Fig 4A). In this regard, bafilomycin A1 treatment increased the low *Lm* signal in *Hdac6*<sup>+/+</sup> DCs to the level observed in *Hdac6*<sup>-/-</sup> cells (Fig 4A).

Confocal fluorescent analysis of *Lm*-infected DCs revealed increased levels of p62 in *Hdac6*<sup>-/-</sup> BMDCs (Fig 4B). *Hdac6*<sup>-/-</sup> BMDCs also showed a higher percentage of p62-*Lm* co-localization than *Hdac6*<sup>+/+</sup> cells, indicating that *Hdac6*<sup>-/-</sup> cells have more number of phagosome-contained bacteria (Fig 4B in accordance with p62 accumulation observed in Figs 3D and 4A). Confocal fluorescent microscopy study of actin and *Lm* revealed more frequent co-localization in *Hdac6*<sup>-/-</sup> than in *Hdac6*<sup>+/+</sup> BMDCs, indicating that more bacteria are at the cytoplasm to form actin rockets in *Hdac6*-deficient cells (Fig 4C). Moreover, more signal of acetylated-cortactin is detected in *Hdac6*<sup>-/-</sup> BMDCs and also higher percentage of acetylated-cortactin-*Lm* co-localization (Fig 4D). These data could explain the accumulation of p62 and the delay in phagocytic vesicle fusion observed in *Hdac6*<sup>-/-</sup> BMDCs, necessary to degrade phagocytosed *Lm*.





**Fig 4. *Hdac6*<sup>-/-</sup> BMDCs accumulate higher levels of p62.** A) Left panels: The charts show geometric means of p62 and *Lm* gated in the MHCII<sup>+</sup>CD11c<sup>+</sup> population of *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> BMDCs without infection (NI) and at 6 hpi, with and without bafilomycin A1 treatment. The representative histograms on the right show p62 and *Lm* with and without bafilomycin A1. \*\*\**p*≤0.001, \*\**p*≤0.01, ns>0.05 non-significant; *n* = 6. B) Confocal microscopy analysis of p62-*Lm* co-localization in *Lm*-infected *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> BMDCs at 6 hpi. Panels show DAPI (blue), *Lm* (red), p62 (green), and merged views of the three channels, with magnified views of the boxed areas. Yellow indicates p62-*Lm* co-localization.

Scale bars 20  $\mu\text{m}$  (main panels) and 10  $\mu\text{m}$  (magnified views). *Right panel:* The chart shows ImarisCell Module analysis of the number of cells and the number of bacteria per cell in all pictures (10 pictures per genotype). Co-localization percentages were obtained by measuring the p62 channel on the bacterial surface using a threshold of 100. The statistical analysis of Imaris quantifications corresponds to the percentage of p62-*Lm* co-localization at 6 hpi. \*  $p \leq 0.05$ ;  $n = 10$ . C) Confocal microscopy analysis of actin-*Lm* co-localization in *Lm*-infected *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> BMDCs at 6 hpi. Panels show DAPI (blue), *Lm* (green),  $\beta$ -actin (red), and merged views of the three channels, with magnified views of the boxed areas. Yellow indicates  $\beta$ -actin-*Lm* co-localization. Scale bars 20  $\mu\text{m}$  (main panels) and 10  $\mu\text{m}$  (magnified views). *Right panel:* The chart shows ImarisCell Module analysis of the number of cells and the number of bacteria per cell in all pictures (10 pictures per genotype). Co-localization percentages were obtained by measuring the actin channel on the bacterial surface using a threshold of 40.6. The statistical analysis of Imaris quantifications corresponds to the percentage of actin-*Lm* co-localization at 6 hpi. \*\*\*  $p \leq 0.001$ ;  $n = 10$ . D) Confocal microscopy analysis of acetylated cortactin-*Lm* co-localization in *Lm*-infected *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> BMDCs at 6 hpi. Panels show DAPI (blue), *Lm* (red), acetylated cortactin (green), and merged views of the three channels, with magnified views of the boxed areas. Yellow indicates acetylated cortactin-*Lm* co-localization. Scale bars 20  $\mu\text{m}$  (main panels) and 10  $\mu\text{m}$  (magnified views). *Right panel:* The chart shows ImarisCell Module analysis of the number of cells and the number of bacteria per cell in all pictures (10 pictures per genotype). Co-localization percentages were obtained by measuring the acetylated cortactin channel on the bacterial surface using a threshold of 184. The statistical analysis of Imaris quantifications corresponds to the percentage of acetylated cortactin-*Lm* co-localization at 6 hpi. \*\*\*  $p \leq 0.001$ ;  $n = 10$ .

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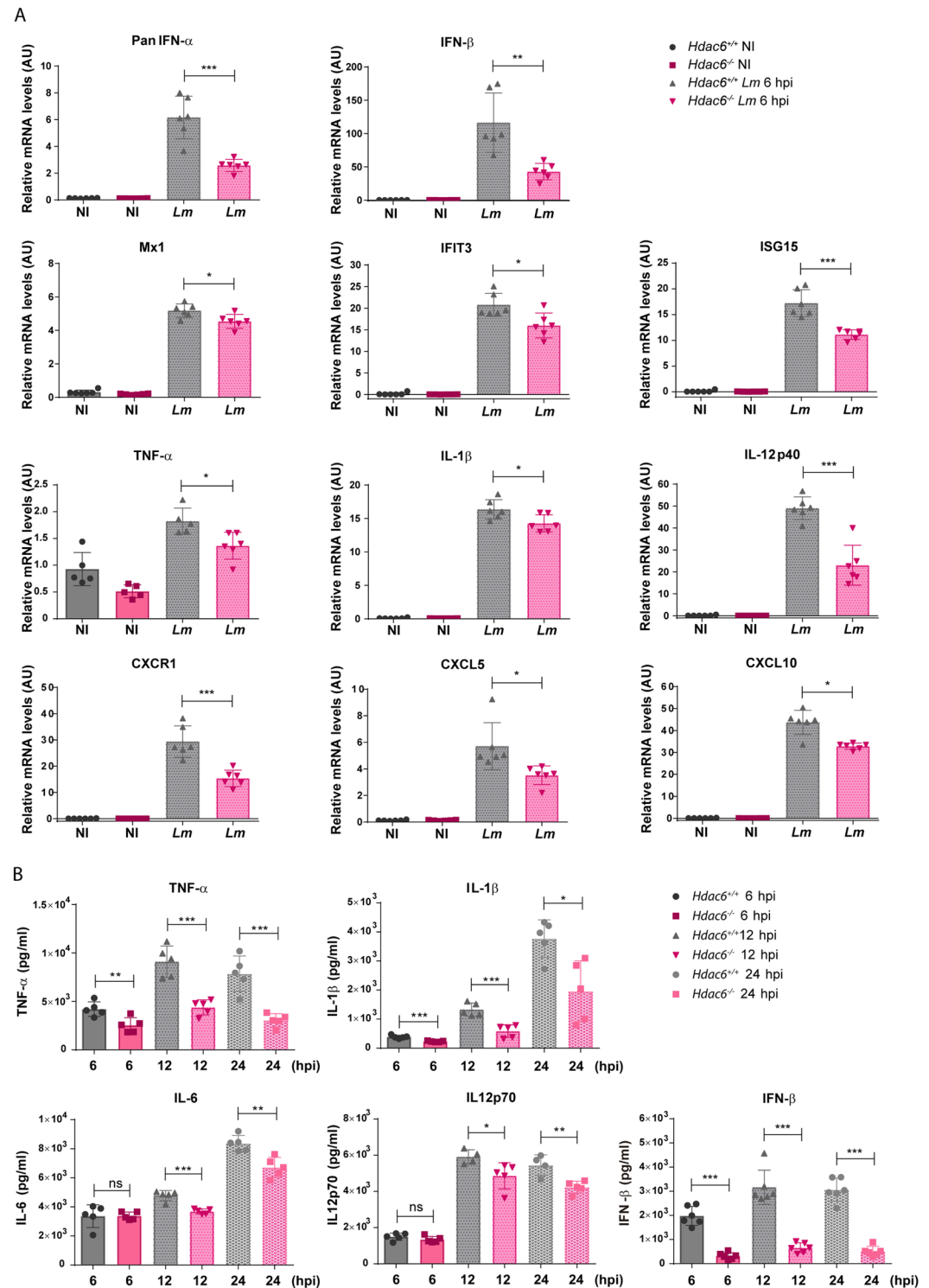
### Defective pro-inflammatory cytokine response to *Lm* in *Hdac6*<sup>-/-</sup> BMDCs

The effect of HDAC6 on the response of BMDCs to *Lm* was evaluated by measuring pro-inflammatory cytokine gene induction. The relative mRNA levels of type I interferons (interferons  $\alpha$  and  $\beta$ ) were lower in *Hdac6*<sup>-/-</sup> BMDCs at 6 hpi (Fig 5A). Accordingly, expression of downstream interferon-response genes such as Mx1, IFIT3, and ISG15 was also lower in *Hdac6*<sup>-/-</sup> BMDCs (Fig 5A). Lack of HDAC6 also decreased the relative mRNA levels of the pro-inflammatory cytokines TNF $\alpha$ , IL-1 $\beta$  and IL12p40, indicating impaired cytokine activation after infection (Fig 5A). Similarly, *Hdac6*<sup>-/-</sup> DCs expressed lower levels than their *Hdac6*<sup>+/+</sup> counterparts of the chemokine receptor CXCR1 and chemokines CXCL5 and CXCL10 (Fig 5A). These data demonstrate that *Hdac6*-deficient DCs have a weakened activation response to *Lm* infection at 6 hpi, which suggests a defect in bacterial clearance, consistent with the increased bacterial load in these cells. To confirm these data, we monitored pro-inflammatory cytokines and IFN- $\beta$  in the supernatants of *Lm*-infected DCs. Early after infection, TNF $\alpha$ , IL-1 $\beta$ , IL-6, IL12p70 and IFN- $\beta$  levels were lower in supernatants from *Hdac6*<sup>-/-</sup> cells than in those from *Hdac6*<sup>+/+</sup> cells, and this difference held at 12 and 24 hpi (Fig 5B). To exclude a defect in cytokine secretion, we compared cytokine levels in supernatants (S) with the levels in supernatants plus their corresponding cell pellets (S+P). Both analyses showed decreased cytokine levels in *Hdac6*<sup>-/-</sup> cells, indicating an impaired antibacterial response in *Hdac6*-deficient DCs (S4 Fig).

Measurement of nitrite in supernatants of infected-BMDCs revealed higher nitric oxide production by *Hdac6*<sup>+/+</sup> DCs than in *Hdac6*<sup>-/-</sup> DCs at 24 hpi (Fig 6A). In agreement, western blot revealed lower levels of inducible nitric oxide synthase (iNOS) in *Hdac6*<sup>-/-</sup> BMDCs at 4 and 6 hpi (Fig 6B), indicating a delay of the enzyme induction in *Hdac6*<sup>-/-</sup> BMDCs. Likewise, flow cytometry after exposure of DCs to live or heat-killed *L. monocytogenes* (HKLM) revealed higher expression of iNOS in *Hdac6*<sup>+/+</sup> BMDCs in both cases (Fig 6C). These data support the involvement of HDAC6 in the activation of DC-mediated iNOS microbicidal responses to *Lm* infection and in the clearance of this intracellular pathogen.

### *Hdac6*<sup>-/-</sup> BMDCs show defective activation of Toll-like receptor signalling pathway

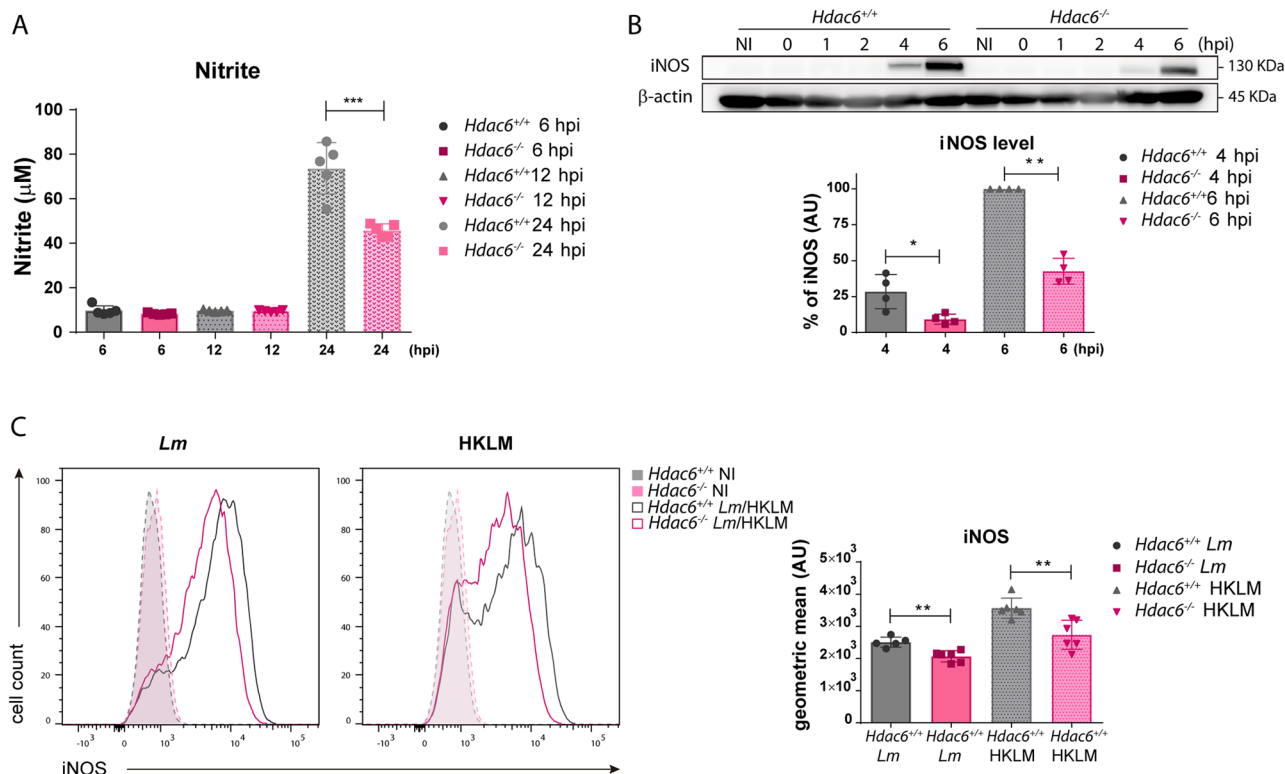
The diminished activation response against *Lm* in *Hdac6*<sup>-/-</sup> BMDCs is consistent with impaired TLR-related signalling. To investigate this question, we determined the phosphorylation levels



**Fig 5. Defective pro-inflammatory cytokine response to *Lm* in *Hdac6*<sup>-/-</sup> BMDCs.** A) PCR analysis of type-I interferons (PanIFN- $\alpha$  and IFN- $\beta$ ), interferon downstream proteins (Mx1, IFIT3 and ISG15), pro-inflammatory cytokines (TNF- $\alpha$ , IL-1 $\beta$  and IL-12p40) chemokine receptor (CXCR1) and chemokines (CXCL5 and CXCL10) of *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> BMDCs non-infected (NI) and infected with *Lm* at 6 hpi (arbitrary units). \*\*\* $p \leq 0.001$ , \*\* $p \leq 0.01$ , \* $p \leq 0.05$ ;  $n = 5-6$ . B) ELISA analysis of the pro-inflammatory cytokines TNF- $\alpha$ , IL-1 $\beta$ , IL-6 and IL12p70 (pg/ml) and IFN- $\beta$  in supernatants of *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> BMDCs at 6, 12 and 24 hpi with *Lm*. \*\*\* $p \leq 0.001$ , \*\* $p \leq 0.01$ , \* $p \leq 0.05$  ns>0.05 non-significant;  $n = 5-6$ .

<https://doi.org/10.1371/journal.ppat.1006799.g005>





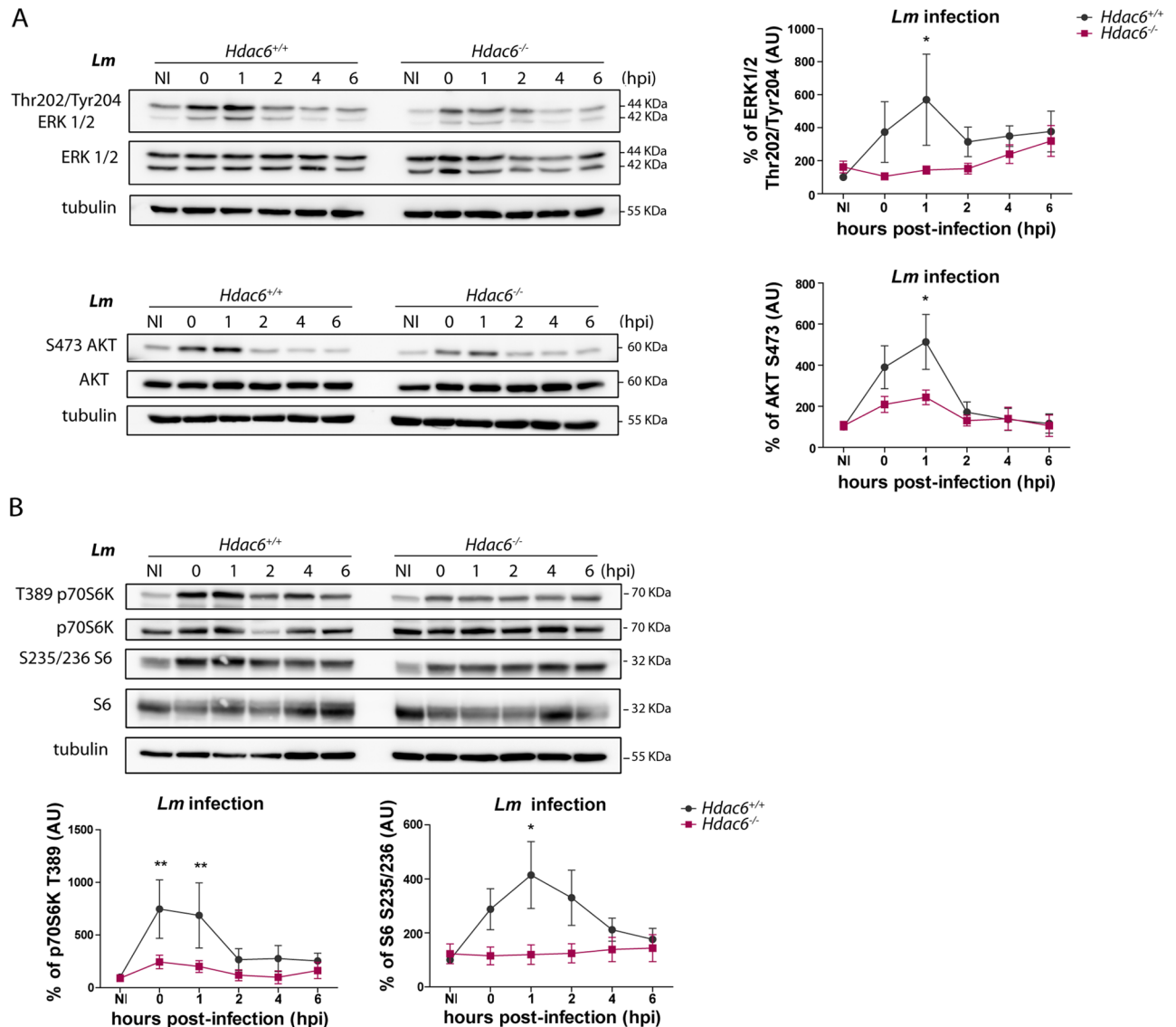
**Fig 6. Defective iNOS response to *Lm* in *Hdac6*<sup>-/-</sup> BMDCs.** A) *Lm*-activated iNOS activity. Nitrite levels in supernatants of *Lm*-infected BMDCs at 6, 12 and 24 hpi. \*\*\* $p \leq 0.001$ ;  $n = 5$ . B) Western-blot analysis of iNOS induction over the time-course of *Lm* infection.  $\beta$ -actin was used as a loading control (top panel). The chart shows quantification of iNOS at 4 and 6 hpi. \*\*  $p \leq 0.01$ , \*  $p \leq 0.05$ ;  $n = 4$  (lower panel). C) The panel shows representative histograms of iNOS expressed by *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> BMDCs after exposure to live *Lm* or HKLM for 24 h (left). The right chart shows the geometric mean of iNOS expression. Non-infected (NI) BMDCs were used as a control of iNOS induction. \*\* $p \leq 0.01$ ;  $n = 6$ .

<https://doi.org/10.1371/journal.ppat.1006799.g006>

of TLR downstream mediators by western blot. Compared with *Hdac6*<sup>+/+</sup> BMDCs, *Hdac6*<sup>-/-</sup> BMDCs showed weaker phosphorylation signals for ERK and AKT after *Lm* infection (Fig 7A). We next examined the effect of HDAC6 deficiency on TLR-signalling pathways using other TLR stimuli, including HKLM and LPS. AKT phosphorylation in *Hdac6*<sup>-/-</sup> BMDCs was decreased after LPS or HKLM treatment compared to *Hdac6*<sup>+/+</sup>, confirming defective TLR activation (S5 Fig part A). These effects are not related to a defect in *Lm*-induced transcriptional induction since mRNA levels of different *Lm*-related TLRs (TLR1, 2, and 6) were similar in *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> BMDCs (S5 Fig part B). Moreover, *Hdac6*<sup>-/-</sup> BMDCs showed weaker phosphorylation of mTORC1 pathway proteins (mTORC1 downstream substrates p70S6K and S6), consistent with a less pronounced pro-inflammatory response after TLR-activation by pathogen-associated molecular patterns (PAMPs) (Fig 7B).

To determine if *Hdac6*<sup>-/-</sup> BMDCs showed a similarly defective response to other TLR agonists, we first examined secretion of pro-inflammatory cytokines in response to agonists of TLR1-2 (Pam3GSK4), TLR-4 (LPS), TLR-7-9 (Imiquimod), and multiple TLRs (heat-killed *Salmonella* Typhimurium; HKST). *Hdac6*<sup>-/-</sup> BMDCs showed a defective cytokine response to these stimuli, determined from the release of TNF $\alpha$ , IL-6, IL-1 $\beta$  and IL12p70 (Fig 8A–8D).

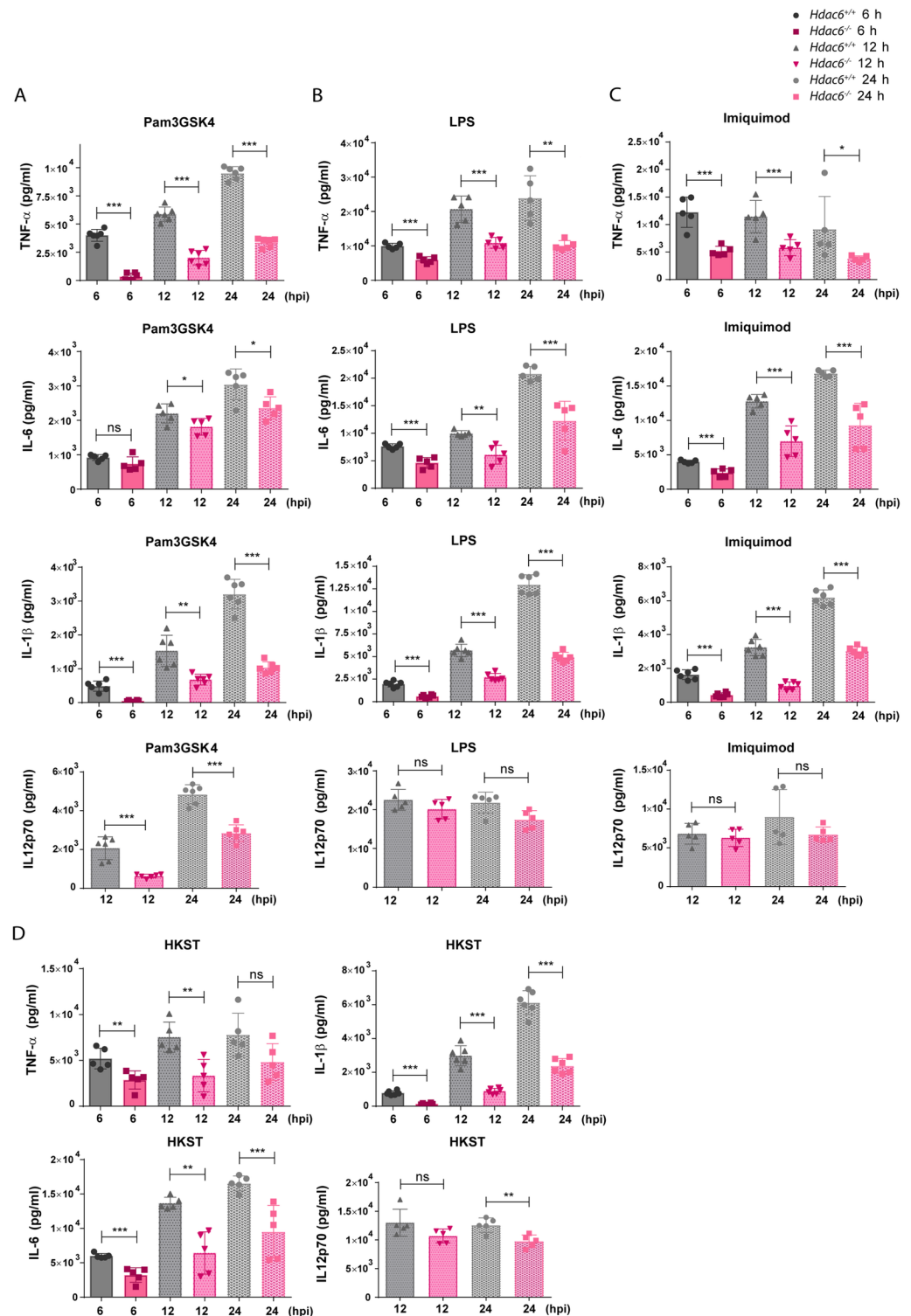
To assess the pro-inflammatory cytokine response to TLR3 and TLR5 ligands, we generated Fms-related tyrosine kinase 3 ligand dendritic cells (FLT3L-DCs). Differentiation with the cytokine FLT3L yielded similar percentages of CD24<sup>+</sup> and CD24<sup>-</sup> subpopulations (CD11c<sup>+</sup>CD11b<sup>+</sup>B220<sup>-</sup>CD24<sup>+</sup> and CD11c<sup>+</sup>CD11b<sup>+</sup>B220<sup>-</sup>CD24<sup>-</sup>, respectively) from *Hdac6*<sup>+/+</sup>



**Fig 7. *Hdac6*<sup>-/-</sup> BMDCs show defective activation of the Toll-like receptor signalling pathway.** A) Western-blot analysis of MAPK activation over the time-course of *Lm* infection in *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> BMDCs. Total and phosphorylated ERK and AKT were detected. Tubulin was used as a loading control (left). Accompanying charts show quantification of pERK/totalERK and pAKT/totalAKT ratios relative to the loading control, ns non-significant; n = 7 (right). B) Western-blot analysis of mTORC1 pathway activation over the time-course of *Lm* infection in *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> BMDCs. Levels of phosphorylated and total p70S6K and S6 were detected. Tubulin was used as a loading control (top panel). Accompanying charts show quantification of pph70S6K/total70S6K (n = 5) and phS6/totalS6 (n = 7) ratios relative to the loading control. \*\* p < 0.01, ns non-significant; (lower panel).

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and *Hdac6*<sup>-/-</sup> DCs, indicating that differentiation is unaffected by HDAC6 absence (S6 Fig part A). The TLR agonists Pam3GSK4 (TLR1-2), Poly(I:C) (TLR3), LPS (TLR4), flagellin (TLR5), Imiquimod (TLR-7-9), *Lm*, HKLM, and HKST (which activates several TLRs simultaneously) elicited similar cytokine secretion profiles in GM-CSF DCs and FLT3L-DCs (Fig 9A and S6 Fig part B compared to Fig 8A–8D). *Hdac6*<sup>-/-</sup> DCs of both derivations showed an impaired cytokine response to each TLR agonist, indicating that HDAC6 likely regulates a common TLR signalling adaptor.



**Fig 8. *Hdac6*<sup>-/-</sup> BMDCs show defective inflammatory cytokine response to Toll-like receptor stimuli.** A) ELISA analysis of the pro-inflammatory cytokines TNFα, IL-1β, IL-6 and IL12p70 (pg/ml) in supernatants of *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> BMDCs after treatment for 6, 12 and 24 h with Pam3GSK4. \*\*\*p<0.001, \*\*p<0.01, \*p<0.05, ns>0.05 non-significant; n = 5–6. B) ELISA analysis of the pro-inflammatory cytokines TNFα, IL-1β, IL-6 and IL12p70 (pg/ml) in supernatants of *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> BMDCs after treatment for 6, 12 and 24 h with LPS. \*\*\*p<0.001, \*\*p<0.01, ns>0.05 non-significant; n = 5–6. C) ELISA analysis of the pro-inflammatory cytokines TNFα, IL-1β, IL-6 and IL12p70

(pg/ml) in supernatants of *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> BMDCs after treatment for 6, 12 and 24 h with Imiquimod. \*\*\**p*≤0.001, \* *p*≤0.05, ns>0.05 non-significant; *n* = 5–6. D) ELISA analysis of the pro-inflammatory cytokines TNFα, IL-1β, IL-6 and IL12p70 (pg/ml) in supernatants of *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> BMDCs after treatment for 6, 12 and 24 h with HKST. \*\*\**p*≤0.001, \*\* *p*≤0.01, ns>0.05 non-significant; *n* = 5–6.

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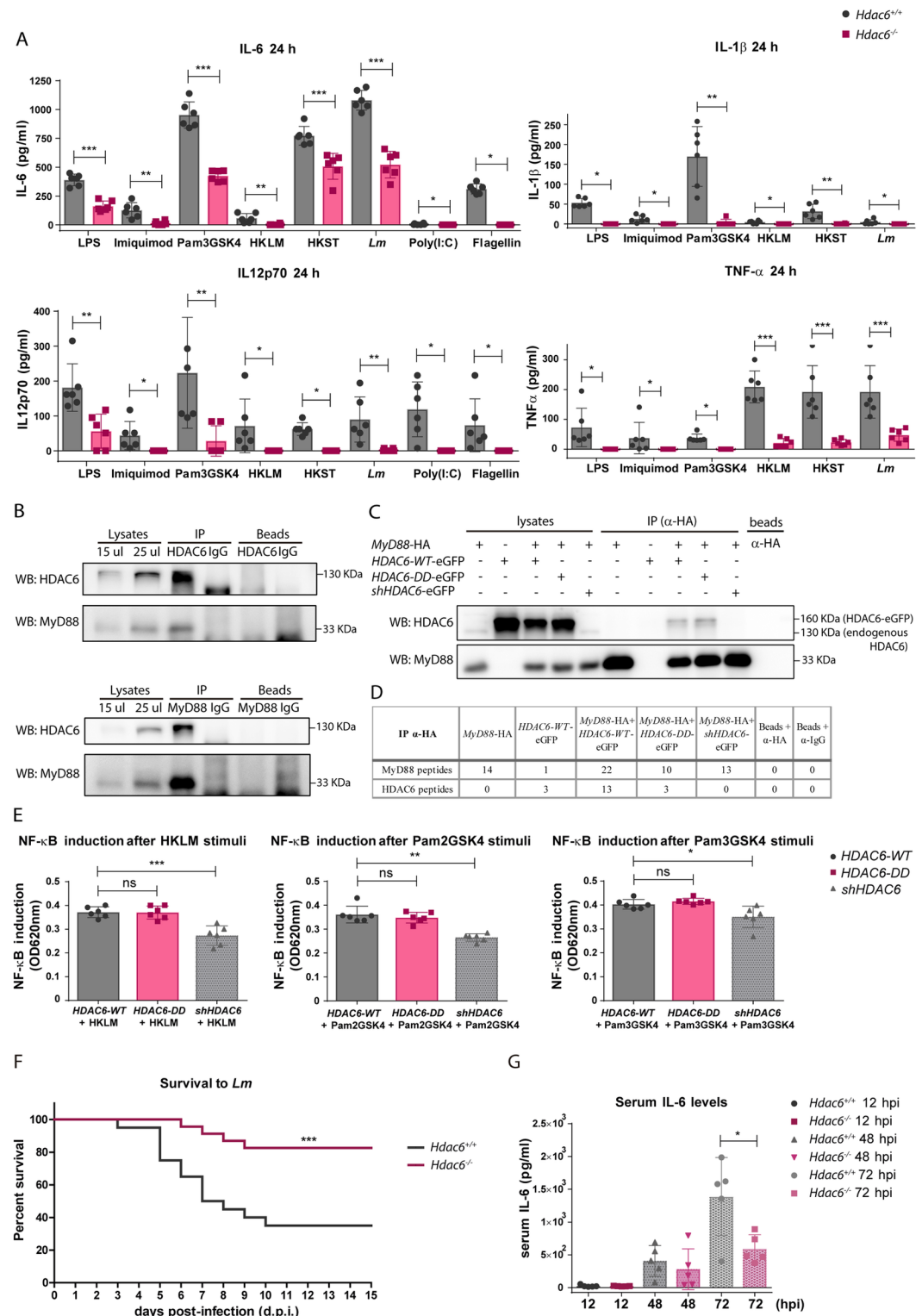
In this view, the TLR adaptor MyD88 participates in the transmission of signals by all TLRs except for TLR3. We decided to study MyD88 levels in a time-course infection with *Lm* by western blot, demonstrating that the quantity of this molecule was the same between *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> DCs and remaining stable during infection (S6 Fig part C). Remarkably, MyD88-HDAC6 molecular association was observed by co-immunoprecipitations of endogenous proteins using human dendritic cells after Pam2GSK4, Pam3GSK4 and HKLM stimulation (Fig 9B). Likewise, the MyD88 immunoprecipitation in MyD88- and HDAC6-overexpressed HEK cell line was also able to co-precipitate HDAC6 (S6 Fig part D). These results were corroborated by mass spectrometry analysis of MyD88 immunoprecipitates; which in addition detected two acetylated peptides corresponding to MyD88 (S6 Fig parts E and F). The same approach was used to determine MyD88-HDAC6 molecular association after TLR-2 activation with HKLM using a constitutively expressed human TLR-2 HEK cell line, rendering the same result (Fig 9C). This association is also maintained using a double-deacetylase domain mutant of HDAC6 (H216A:H611A), called HDAC6-DD, indicating that HDAC6-MyD88 interaction is independent of its catalytic activity (Fig 9C and S6 Fig part D). The knock-down of HDAC6 expression using a small harping plasmid (sh-HDAC6) blocked this interaction (Fig 9C and S6 Fig part D). However, no acetylated peptides could be detected in the mass spectrometry analysis of the MyD88 immunoprecipitation from HKLM-stimulated TLR-2 HEK cell line (Fig 9D). Moreover, assessment of NF-κB induction in TLR-2-expressing HEK cell line after HKLM, Pam2GSK4 and Pam3GSK4 stimulation shows lower activation only in shHDAC6 transfected cells, without affecting the activity of HDAC6-DD-transfected ones (Fig 9E).

Taking into account all these data, HDAC6 associates with the TLR-adaptor molecule MyD88, and the absence of HDAC6 in DCs seems to diminish the TLR-response after a variety of stimuli, underlining the scaffold role of HDAC6 in determining the ability of MyD88 to mediate TLR signalling.

To ascertain the defective TLR-dependent inflammatory response *in vivo* due to the molecular association of MyD88-HDAC6, *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> mice were intravenously injected with a lethal dose of *Lm* (Fig 9F) [44]. A protective effect against *Lm*-induced septic shock was observed from 3 to 10 days post-infection (dpi) (Fig 9F). Accordingly, lower levels of the pro-inflammatory cytokine IL-6 were detected in the serum of *Hdac6*<sup>-/-</sup> mice at 72 hpi, highlighting a reduced systemic cytokine-driven inflammatory response after *Lm* infection in these mice (Fig 9G).

## Discussion

Recent studies have revealed the involvement of HDAC6 in the innate immune response against Influenza Virus A (IVA), Sendai virus (SeV), and vesicular stomatitis virus (VSV) [21–24]. Given the similarities between the innate responses to viruses and intracellular bacteria, this prompted us to investigate the role of HDAC6 in a model of *Lm* infection. In this work, we demonstrated a dual role of HDAC6 in the innate response against *Lm*, not only due to its enzymatic activity but also dependent of its function as a scaffold (Fig 10). Our data clearly demonstrate that *Hdac6*<sup>-/-</sup> BMDCs have an impaired immune response against *Lm* and *S. Typhimurium* infection *in vitro*. Moreover, higher *Lm* titres observed in *Hdac6*<sup>-/-</sup> dendritic cells, M-CSF-derived macrophages and peritoneal macrophages were corroborated during *in*



**Fig 9. Association of HDAC6 with TLR-adaptor MyD88 and its contribution to the inflammatory response to *Lm*.** A) ELISA analysis of the pro-inflammatory cytokines TNF $\alpha$ , IL-1 $\beta$ , IL-6 and IL12p70 (pg/ml) in supernatants of *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> FLT3L-DCs activated with LPS, Imiquimod, Pam3GSK4, HKLM, HKST, *Lm*, Poly(I:C) and flagellin for 24 h. \*\*\*p $\leq$ 0.001, \*\*p $\leq$ 0.01, \*p $\leq$ 0.05; n = 6. B) Immunoprecipitation of endogenous HDAC6 and MyD88 followed by western-blot for both proteins. Immunoprecipitations were carried out using human moDCs after 30 min of stimulation with



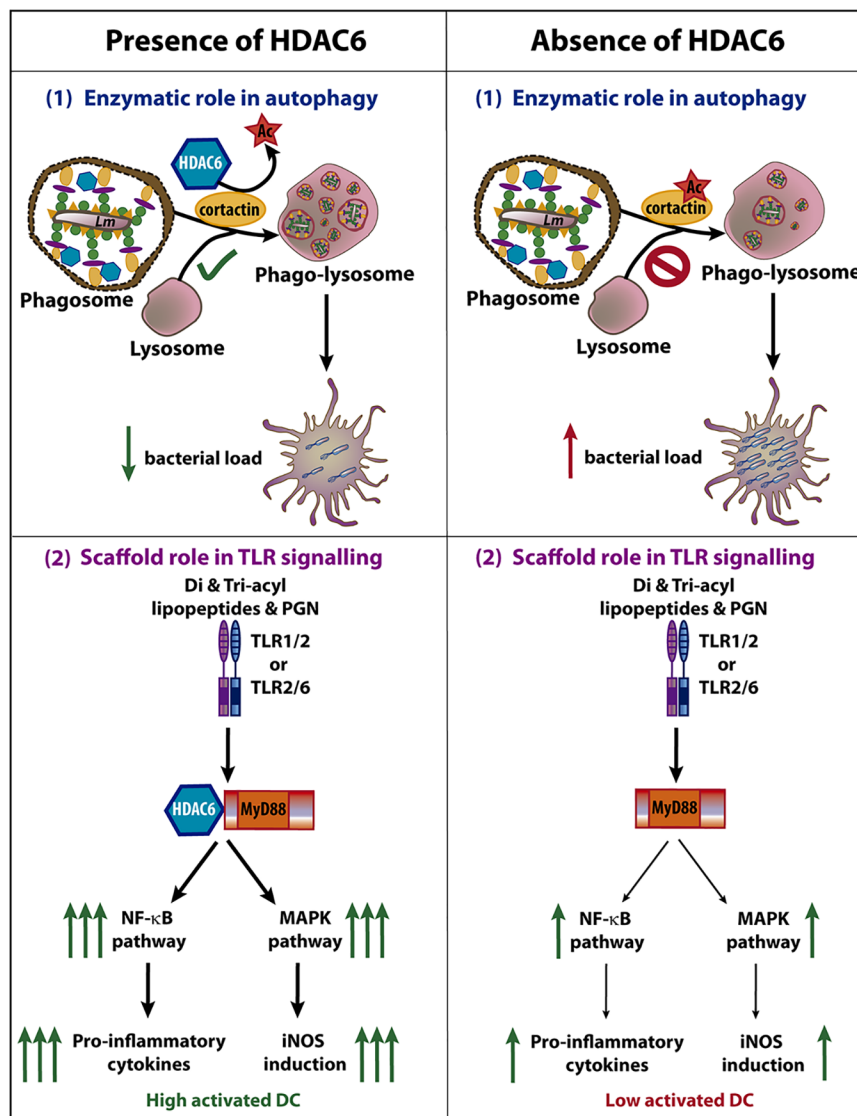
Pam2GSK4, Pam3GSK4 and HKLM. Endogenous HDAC6 (130 KDa) and MyD88 (33 KDa) are indicated at right of western-blot. Similar results were obtained in two independent experiments. C) Immunoprecipitation of HA (MyD88) followed by western-blot for HDAC6 and MyD88. Immunoprecipitations were carried out using different HDAC6-eGFP plasmids co-transfected with MyD88-HA in HEK-Blue hTLR2 cell line after 30 min of stimulation with HKLM. Over-expressed (HDAC6-eGFP, 160 KDa) and endogenous HDAC6 (130 KDa) are indicated at right of western-blot. Similar results were obtained in four independent experiments. D) Immunoprecipitation of HA (MyD88) followed by mass spectrometry analysis. Immunoprecipitations were carried out using different HDAC6-eGFP plasmids co-transfected with MyD88-HA in HEK-Blue hTLR2 cell line after 30 min of stimulation with HKLM. The number of unique MyD88 and HDAC6 peptides is indicated. No acetylated peptides from MyD88 were detected in any sample. Similar results were obtained in four independent experiments. E) Graph of NF- $\kappa$ B induction in transfected HDAC6-WT, HDAC6-DD and *shHDAC6* HEK-Blue hTLR2 cell line after activation with HKLM, Pam2GSK4 and Pam3GSK4 during 8 h. NF- $\kappa$ B induction was calculated by the ratio of the signal of stimulated cells with its corresponding transfected cells in basal condition (without stimuli), \*\*\* $p \leq 0.001$ , \*\* $p \leq 0.01$ , \* $p \leq 0.05$ , ns $>0.05$  non-significant;  $n = 6$ . F) Survival curve to intravenous injection with a lethal dose of *Lm* in *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> is showed. This curve corresponds to two different experiment of survival to *Lm* with a  $n = 24-21$ . \*\*\* $p \leq 0.001$ . G) Pro-inflammatory cytokine IL-6 was measured in sera of *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> mice intravenously injected with a lethal dose of *Lm* at 12, 48 and 72 hpi. \* $p \leq 0.05$ ,  $n = 5$ .

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*vivo* *Lm* infection at 6 hpi in various myeloid subsets of the spleen. The absence of this effect during BMDC *in vitro* infection by the non-intracellular bacteria *S. aureus* and *E. coli* DH5 $\alpha$  indicates that *Hdac6*<sup>-/-</sup> BMDCs are specifically unable to efficiently clear intracellular pathogens. HDAC6 is involved in two of the most important cellular clearance systems, autophagy and ubiquitin-proteasome system (UPS) [9, 13]. In the case of *Lm* and *S. Typhimurium*, the main molecular mechanism for degradation of vesicle-contained bacteria is fusion with lysosomes in a process called autophagy [45–47]. In agreement with this, our data show that impaired phagosome-lysosome fusion underlies the phenotype observed in *Hdac6*<sup>-/-</sup> BMDCs. Unsuccessful fusion depends on acetylated-cortactin in *Hdac6*-deficient cells [9]. A similar mechanism has been described in *Hdac6*-deficient MEFs during quality-control autophagy [9]. We demonstrated that in *Hdac6*<sup>-/-</sup> BMDCs co-localized higher levels of acetylated-cortactin with intracellular *Lm*. The delay in vesicle fusion caused by the acetylation of cortactin, impairs the phagosome-lysosome fusion and provides more opportunities for phagosome-containing bacteria to escape to the cytosol, resulting in the higher bacterial load detected in *Hdac6*<sup>-/-</sup> BMDCs. Based on this experimental evidence, it is conceivable to postulate that the enzymatic activity of HDAC6 on its substrate cortactin controls autophagy of intracellular bacteria for their efficient clearance (Fig 10).

Pharmacological autophagy inhibitors erased the observed differences between *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> BMDCs. Conversely, rapamycin did not overcome the *Hdac6*<sup>-/-</sup> autophagy defect, indicating a defect in phagosome-lysosome fusion. However, other authors have reported opposite observations using pan-HDAC inhibitors or specific inhibitors of HDAC6 during infection of human macrophages with the Gram-negative intracellular pathogens *S. Typhimurium* and *E. coli* [48]. These inhibitors, when added at the time of infection, increase mitochondrial ROS production [48]. However, overnight pre-treatment before infection hampered bacterial clearance and reduced phagocytosis [48]. These data indicate that specific HDAC6 chemical inhibitors can have side-effects, including effects on other HDAC members, potentially interfering with the acetylation of other substrates upstream of cortactin that also have a role during bacterial infection. Our genetic approach unequivocally assigns a specific role to HDAC6 in innate cells during bacterial infection.

Although we observed an impairment in phagosome-lysosome fusion, we cannot rule out an involvement of HDAC6 in the anti-microbial response through its BUZ domain, with a contribution from ubiquitin. The characterized interaction between p62 and HDAC6 through their ubiquitin-binding domains provides a clue about the possible role of ubiquitin in the activation of innate immunity through the recognition of ubiquitinated-molecules [15]. For example, the ubiquitin-binding regions of HDAC6 and p62 are both essential for MyD88



**Fig 10. Dual role of HDAC6 during *Lm* infection in dendritic cells.** The scheme shows the involvement of HDAC6 in two different functions of dendritic cell during *Lm* infection, the autophagy and the TLR signalling. (1) The fusion of phagosome with lysosome is dependent on cortactin and F-actin. The deacetylation of cortactin by HDAC6 allows the correct fusion, followed by an autophagic clearance of *Lm*. The absence of this enzyme delays the fusion of phagosome and lysosome, facilitating *Lm* to escape from phagosome leading to an increased bacterial load. (2) Di- and tri-acyl lipopeptides and peptidoglycan (PGN) of *Lm* are recognized by TLR1/2 or TLR2/6, activating the TLR pathway. HDAC6 is able to interact with the TLR-adaptor protein MyD88 which caused an enhanced down-stream signalling of TLR pathway, increasing NF- $\kappa$ B and MAPK activation. This stronger activation (independent on HDAC6 enzymatic activity) results in higher pro-inflammatory cytokine production and iNOS induction, reinforcing the ability of the DC to combat against this intracellular pathogen. Although the absence of HDAC6 does not fully abolish the activation of the DC, a lower induction of NF- $\kappa$ B and MAPK pathways promotes a lower activation of the anti-bacterial transcriptional program of the DCs. Note that both processes occur during *Lm* infection and the pro-inflammatory cytokines and iNOS induction can impact on the autophagic process. The images in the figure are not scaled.

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aggregation and the downstream activation of MyD88-dependent signal transduction [49]. Furthermore, ubiquitin-binding platforms formed by HDAC6 and p62 are able to interact with interferon stimulated gene 15 (ISG15) to eliminate ISGylated proteins tagged after interferon stimulation by autophagy [50]. HDAC6 is able to bind to either mono- and poly-

ubiquitinated proteins, but shows a preference for proteins modified with k63-linked ubiquitin chains, which share structural similarities with ISG15 [51]. *S. Typhimurium* is decorated with this kind of ubiquitin chain for recognition by host cells, and can be recovered with phagosome proteins to initiate autophagy [52, 53]. Nevertheless, our data demonstrate that autophagy induction does not differ between *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> BMDCs, indicating that this phenotype is due to p62 accumulation in *Hdac6*<sup>-/-</sup> BMDCs as a consequence of impaired phagosome-lysosome fusion. Intact autophagy activation in *Hdac6*<sup>-/-</sup> BMDCs could be explained by compensatory p62 binding to ubiquitinated bacteria in the absence of HDAC6.

Our data also underscore other different function of HDAC6, independent of its enzymatic activity, in innate immune response to intracellular bacteria and various TLR stimuli (Fig 10). Hence, we provide evidence for a dampened inflammatory response in the absence of HDAC6, as shown by lower RNA levels of pro-inflammatory cytokines, chemokines, type-I interferons, and interferon-related proteins in *Hdac6*<sup>-/-</sup> BMDCs than in *Hdac6*<sup>+/+</sup> cells at 6 hpi, as well as the lower pro-inflammatory cytokine production and IFN- $\beta$  secretion by infected *Hdac6*<sup>-/-</sup> BMDCs from 6 to 24 hpi. Moreover, *Hdac6*<sup>-/-</sup> BMDCs showed diminished iNOS induction at 6 hpi associated with low nitrite production and iNOS expression at longer times of *Lm* infection (24 hpi). These results agree with a lower phosphorylation of the MAPK pathway after *Lm* infection in *Hdac6*<sup>-/-</sup> dendritic cells, controlling the activation of AP-1 family transcription factors, which is necessary to switch inflammatory responses on [54]. In addition, the lower phosphorylation of mTORC1 pathway components in *Hdac6*<sup>-/-</sup> DCs is consistent with a lower pro-inflammatory response, as reported in trained macrophages and dendritic cells, in which a metabolic switch to glycolysis has been described [55]. These data may indicate that HDAC6 also appears to play a role in the activation of mTOR pathway after *Lm* infection to initiate the antibacterial transcriptional response to combat this pathogen. In summary, our results reveal a defect in DC activation after *Lm* entry.

Remarkably, this impaired anti-inflammatory response in *Hdac6*<sup>-/-</sup> BMDCs was also observed with other TLR-agonists such as LPS, Imiquimod, poly(I:C) and Pam3GSK4, highlighting HDAC6 as an important player in TLR signalling activation. Broad-spectrum HDAC inhibitors such as TSA exert immunosuppressive effects [56]. Genome-wide expression profiling arrays have revealed that 60% of genes transcriptionally increased by TLR2 or TLR4 stimulation are inhibited in TSA-treated cells, whereas 16% of genes are potentiated [56]. However, these observations do not provide any demonstrative evidence for a specific role of HDAC6, since other HDACs may also be involved.

Because GM-CSF-derived DCs express low levels of TLR3 and 5 in the membrane, we stimulated FLT3-L-derived DCs with poly(I:C) and flagellin to measure pro-inflammatory cytokines [57, 58], noting maintained failure in the inflammatory response in *Hdac6*<sup>-/-</sup> cells. Moreover, we detected impaired responses to PAMPs activation in GM-CSF-derived and FLT3L-derived *Hdac6*<sup>-/-</sup> DCs. In addition, all TLRs except for TLR3 signal through the adaptor MyD88, and the result obtained with the TLR3 ligand poly(I:C) was similar to that showed for the rest of TLR stimuli, thereby indicating that the TLR3-response is also affected by absence of HDAC6. In this regard, these data are in agreement with a recent study showing that acetylated retinoic-acid-inducible gene-1 (RIG-1) makes *Hdac6*<sup>-/-</sup> cells less sensitive to the presence of RNA viruses, resulting in a higher viral titre [59]. While this mechanism could explain the difference between the response of *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> FLT3L-DCs to TLR3 stimulation, the deficient activation by other TLRs in *Hdac6*<sup>-/-</sup> DCs also requires an explanation. In this respect, we demonstrate the molecular association of HDAC6 with MyD88 with endogenous proteins and in an overexpression system. Conceivably, the depletion of HDAC6 and therefore prevention of HDAC6-MyD88 binding, could inhibit TLR-2-signalling pathway activation, which is in accordance with a lower NF- $\kappa$ B induction measured in *Hdac6*<sup>-/-</sup> cells after various



TLR-2 agonist stimulation. A diminished NF- $\kappa$ B induction in *Hdac6*<sup>-/-</sup> cells could explain a reduced initiation of the pro-inflammatory response observed in *Hdac6*<sup>-/-</sup> dendritic cells, needed to alert the innate and adaptive immune response to *Lm*. However, NF- $\kappa$ B activity of HDAC6-DD-transfected HEK cell line after TLR-2 stimuli did not display any significant change compared to HDAC6-WT-transfected ones, supporting that enzymatic activity of HDAC6 is not involved in this signalling pathway. Two acetylated peptides corresponding to MyD88 have been found in basal condition in HEK transfected with HDAC6-DD and shHDAC6 constructions, which are different from the residue previously described in MyD88 [60]. However, no changes in the acetylation marks on MyD88 were detected after HKLM incubation, highlighting the scaffold role of HDAC6 in the proper activation of TLR-signalling pathway (Fig 10). Unexpectedly, a protective effect against systemic infection to a lethal dose of *Lm* were observed in *Hdac6*<sup>-/-</sup> mice. The reduced level of the inflammatory cytokine IL-6 detected in *Hdac6*<sup>-/-</sup> mice are in accordance with its higher resistance to *Lm* infection. The defective systemic inflammatory response after *Lm* infection of *Hdac6*<sup>-/-</sup> mice may indicate an impaired TLR-response in the absence of HDAC6 and might therefore be attributed to the absence of the molecular association of MyD88 and HDAC6. In this regard, mice resistance to *Lm* infection can be mediated by sequential MyD88-independent and -dependent responses [61–64]. However, the role of TLR-2 during *Lm* infection does not appear to be clear enough [62, 63]. On one hand, *Tlr-2*<sup>-/-</sup> mice display a deficit in circulating TNF- $\alpha$  and IL-12p40 production during intravenously injected *Lm* infection combined with a lower mice survival and increased bacterial burden in the liver [61, 63]. Other authors have obtained similar resistance to intraperitoneal-injected *Lm* infection between *Tlr-2*<sup>-/-</sup> and *Tlr-2*<sup>+/+</sup> mice, indicating that different inoculation routes of bacteria may render different immune outcomes [62]. Although handling and direct killing of *Lm* by activated macrophages can be mediated by TLR-2- and MyD88-independent mechanisms, the role of TLR-signalling has been observed necessary for nitric oxide and cytokine production [61, 63]. In fact, MyD88 not only works as TLR-adaptor protein, but also as adaptor of IL-1 and IL-18 receptors, both cytokine responses affected in *Lm*-MyD88<sup>-/-</sup> mice [62, 63, 65].

Overall, our data support a dual role for HDAC6 in the regulation of innate immunity against intracellular bacteria. An increased bacterial load in different *Hdac6*<sup>-/-</sup> myeloid cells can be explained by the autophagy mechanism, where a permanently acetylated cortactin may impair the phagosome-lysosome fusion, necessary for the clearance of this pathogen. Our experiments also show the importance of HDAC6 in DC activation, uncovering a novel mechanism of HDAC6 action mediated by the appropriate signalling via the TLR pathway, due to the association of HDAC6 with the TLR-adaptor protein MyD88. This molecular association seems to be required for a response to TLR stimuli to initiate the inflammatory response of an activated dendritic cell. Taken together, both HDAC6 functions described in this manuscript, reinforce the importance of this molecule to combat intracellular bacteria as *Lm* by autophagy and to completely activate the inflammatory response after TLR activation.

## Materials and methods

### Ethical statement

Mice were housed under specific pathogen-free conditions at the Centro Nacional de Investigaciones Cardiovasculares Carlos III (CNIC), and experiments were approved by the CNIC Ethical Committee for Animal Welfare and by the Spanish Ministry of Agriculture, Food, and the Environment. Animal care and animal procedures license were reviewed and approved by the local Ethics Committee for Basic research at the CNIC Ethical Committee for Animal Welfare and the Órgano Encargado del Bienestar Animal (OEBA) del Gabinete Veterinario de la

Universidad Autónoma de Madrid (UAM). This committee approved the document with an associated identification number PROEX 158/15 (CNIC 04/15).

Buffy coats of healthy donors were received from the Blood Transfusion Center of Comunidad de Madrid, and all donors signed their consent for the use of samples for research purposes. All the procedures using primary human cells were approved by the Ethics Committee of the Hospital Universitario de la Princesa.

## Mice

*HDAC6*<sup>-/-</sup> mice were generated through targeting of exons from 10 to 13 by inserting a neomycin (Neo) and zeocin (Zeo) cassette, resulting in the disruption of the first catalytic domain of HDAC6 [66]. These mice were intercrossed on a C57BL/6 background to generate sex and age matched wild-type (*wt*) and knockout.

## Bacteria strains

We used the *Listeria monocytogenes* EGD (BUG 600) strain, provided by Dr. Esteban Veiga (Centro Nacional de Biotecnología, CNB, Madrid). *Staphylococcus aureus* 132 and *Escherichia coli* K12, strain DH5 $\alpha$ , were purchased from Invitrogen. BUG600 and *S. aureus* bacteria were grown in BHI broth. RFP-expressing *Listeria monocytogenes* (RFP-*Lm*) was provided by Dr. Carlos Ardavín's laboratory (Centro Nacional de Biotecnología, CNB, Madrid). *Salmonella enterica* serovar Thyphimurium strain SL1344 was provided by Dr. J. Garaude (Centro Nacional de Investigaciones Cardiovasculares, CNIC, Madrid). SL1344 and DH5 $\alpha$  bacteria were grown in LB broth supplemented with 50  $\mu$ g/ml streptomycin (Sigma). For phagocytosis experiments, *Lm* and *S. aureus* were grown overnight in Brain Herat Infusion (BHI) broth and *E. coli* and *S. Thyphimurium* in Luria-Bertani (LB) broth with shaking, diluted 1/50, and grown until log-phase (optical density 0.8–1.2 at 600 nm) without shaking. Bacteria were washed with phosphate-buffered saline (PBS) to remove LB salts before addition to cells.

## Cell culture

The HEK293T cell line (ATCC) was cultured in DMEM medium (Sigma) containing 10% FBS (Invitrogen), 2 mM L-glutamine, 100 mg/ml penicillin and 100 mg/ml streptomycin. HEK Blue hTLR2 cell line (Invivogen), the HEK293 cell line expressing human TLR2, CD14 and NF- $\kappa$ B-SEAP (secreted embryonic alkaline phosphatase) reporter gene was cultured in DMEM medium (Sigma) containing 10% FBS (Invitrogen), 2 mM L-glutamine, 100  $\mu$ g/ml Normocin (Invivogen) and 1X HEK-Blue Selection (Invitrogen).

## Generation of bone marrow-derived dendritic cells (GM-CSF) and macrophages (M-CSF)

Mouse primary dendritic cells (BMDCs) and macrophages (BMDMs) were obtained from bone marrow cell suspensions after culture on non-treated 150-mm Petri dishes in complete RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, 100 mg/ml penicillin, 100 mg/ml streptomycin, 50 mM 2-ME, and 20 ng/ml granulocyte-macrophage colony-stimulating factor (GM-CSF, PeproTech, London, U.K.) for BMDCs and macrophage colony-stimulating factor (30% mycoplasma-free L929 cell supernatant, NCBI Biosample accession number SAMN00155972) for BMDMs. BMDCs were collected at day 9 and BMDCs were characterized as CD11c<sup>+</sup>MHC-II<sup>+</sup>Gr-1<sup>-</sup> cells by flow cytometry. BMDMs were collected at day 6 and BMDMs were characterized as CD11b<sup>+</sup>F4/80<sup>+</sup> or CD11b<sup>+</sup>CD64<sup>+</sup> cells.

### Generation of bone marrow-derived dendritic cells (FLT3L)

Bone marrow cell suspensions were culture on treated 6 well plates in complete RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, 100 mg/ml penicillin, 100 mg/ml streptomycin, 50 mM 2-ME, and 150 ng/ml (FLT3L, PeproTech, London, U.K.). After 9–11 days of differentiation cells were collected to be characterized by flow cytometry as CD11c<sup>+</sup>B220<sup>−</sup>CD11b<sup>+</sup>CD24<sup>−</sup> (60% of the culture) and CD11c<sup>+</sup>B220<sup>−</sup>CD11b<sup>+</sup>CD24<sup>+</sup> (40%).

### Obtainment of thioglycollate-elicited macrophages (TEMs)

Mice received peritoneal injections with 1ml 4% TG. The peritoneal exudate was collected after 4 days and cultured in complete RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, 100 mg/ml penicillin, 100 mg/ml streptomycin, and 50 mM 2-ME. To enrich the culture for macrophages, non-adherent cells were eliminated after a few hours by washing five times with warm PBS and gentle swirling.

### Obtainment of human monocyte-derived dendritic cells (moDCs)

Peripheral blood mononuclear cells (PBMCs) from Buffy coats of healthy donors were isolated using Biocoll separating solution (Millipore) by centrifugation at 700 g 30 min at RT. Monocytes were purified from peripheral blood mononuclear cells (PBMCs) by an adherence step at 37°C in incomplete RPMI 1640 medium during 1 h. Non-adherent cells were removed and adherent monocytes were washed three times with warm 1xPBS to remove residual PBMCs. Monocytes were cultured in complete RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, 100 mg/ml penicillin, 100 mg/ml streptomycin, 500 U/ml IL-4 (R&D) and 500 U/ml GM-CSF (Immunotools) for 6 days. Fresh medium and cytokines were added every 48 hours to differentiate monocytes to immature human dendritic cells. Cells were characterized by flow cytometry as HLA-DR<sup>+</sup>CD3<sup>−</sup>DC-SIGN<sup>+</sup>CD14<sup>+</sup>CD11c<sup>+</sup>. Activation of dendritic cells was induced with Pam2GSK4, Pam3GSK4 and HKLM for 30 min (Invivogen).

### *In vitro* *Lm*-infection of BMDCs, BMDMs and TEMs

Cells were incubated with *Lm* and assessed for survival to gentamicin exposure [67]. Cells were infected with *Lm* at a multiplicity of infection (MOI) of 10 for 30 min at 37°C. To determine the number of bacteria entering the cells, extracellular bacteria were killed by treatment with 100 µg/ml gentamicin (Sigma-Aldrich, St. Louis, MO) for an additional 30 min at 37°C. Then, infected cells were washed with PBS three times and lysed with 0.05% Triton X-100 (Sigma-Aldrich, St. Louis, MO) in distilled water. Serial dilutions were seeded on brain-heart infusion (BHI) agar plates and CFUs were counted after 36 hours.

### *In vivo* *Lm* systemic infections

*Hdac6*<sup>+/+</sup> and *Hdac6*<sup>−/−</sup> were intravenously injected with *Listeria monocytogenes* EGD (125,000 CFUs/mouse) using a 29-gauge needle. For survival experiments mice were monitored twice a day in order to detect casualties during 15 days of infection.

### Determination of CFUs in target organs

After 12, 24, 48 and 72 hpi, mice were perfused with 1X PBS to clean blood form organs and spleens and livers were weight. To determine bacterial load, spleens and livers were digested with 0.1 mg/ml type IV collagenase and 0.5 mg/ml DNase I (Roche, Mannheim, Germany) for 30 min at 37°C. After digestion, organs were homogenized in 70 µm filters and red blood cells were lysed with ammonium chloride potassium lysis buffer (ACK, Sigma). Splenic cell

suspensions were resuspended in PBS and cells were counted. Serial dilutions were grown on BHI agar plates. CFUs were counted after 36 hours of incubation at 37°C. CFUs were calculated by cell number and by gram per organ.

### Antibodies and reagents

Antibodies were used in western blotting, flow cytometry and immunofluorescence; detailed information is available in [S1 Table](#). Poly-L-lysine (PLL) was purchased from Sigma. Phalloidin-Alexa488 and 647 were from BD Biosciences. Zenon Alexa Fluor 488 rabbit IgG labelling kit, DAPI and Prolong Gold anti-fade mounting medium were from Thermofisher Scientific. Anti-human CD3 antibody (T3b hybridoma) was generated in Dr. F. Sánchez-Madrid laboratory (Hospital Universitario de la Princesa, HUP, Madrid) [68]. Rapamycin, bafilomycin A1, 3-MA, cloroquine, NH<sub>4</sub>Cl, 1400W and DPI were from Sigma-Aldrich.

### Gene overexpression and silencing

HEK293 cells were co-transfected with plasmids encoding human MyD88 fused to the HA-tag (Addgene plasmid #12287) together with plasmids encoding *HDAC6-WT* or double deacetylase domain mutant DD (mutated human HDAC6-H216A/H611A) fused to the eGFP tag (*HDAC6-WT* and *HDAC6-DD* have been previously described [26]). When indicated, cells were co-transfected with the appropriate small harping RNA plasmid pLVX-IRES-ZsGreen1, where *shHDAC6-2049* (TRCN0000004842) was cloned between BamH1 and EcoR1 sites. Cells were transfected using Lipofectamine 2000 (Invitrogen). Experiments were performed after 24 h after transfection.

### RNA extraction and real-time quantitative PCR

RNA from mouse BMDCs was isolated with the QIAGEN RNeasy Kit (Qiagen). Residual DNA contamination was removed with the Turbo DNA-free Kit (Ambion). Total RNA (1–2 µg) was reverse transcribed to cDNA with a Reverse Transcription Kit (Applied Biosystems). Quantitative PCR was then performed in an AB7900-384 thermocycler (Applied Biosystem) using SYBR Green master mix (Applied Biosystems, Warrington, UK) as the reporter. Expression levels of target genes were normalized to the expression of housekeeping genes  $\beta$ -actin, GAPDH,  $\beta$ 2-microglobulin and Yhwaz (tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein,  $\zeta$ ) for presentation of relative mRNA levels. Data were analysed with Biogazelle qBasePlus version 2.3 (Biogazelle) and graphs are represented as a normalized expression scaled to average of all samples. Gene-specific primers used are listed in [S2 Table](#).

### Soluble embryonic alkaline phosphatase (SEAP)-NF- $\kappa$ B detection

50.000 transfected HEK-Blue hTLR2 cells with different HDAC6 constructions were place in bottom p96 well plates resuspended in HEK-Blue Detection medium (Invivogen) without stimulus (negative control) and with TLR-2 agonists (HKLM stimulus, MOI = 10). After 8–12 h of incubation, SEAP activity was measured by optical density at 620 nm with a microplate reader. To calculate the NF- $\kappa$ B induction, the signal obtained from each mutant condition without stimuli (background) was depleted of the signal of each condition of activation with Pam2GSK4, Pam3GSK4 or HKLM.

### ELISAs and nitrite measurement

Cytokine and NO production was analysed in the supernatants of BMDC cultures at 6, 12 and 24 h after stimulation with *Lm*, heat-killed *Listeria monocytogenes* (HKLM), heat-killed

*Salmonella* Typhimurium (HKST), Pam3CSK4, Flagellin, Imiquimod, polyinosinic-polycytidylic acid (Poly(I:C)) (InvivoGen, San Diego, CA), or LPS from *Escherichia coli* (Sigma-Aldrich). TNF- $\alpha$  and IL12p70 were analysed with OptEIA ELISA kits (BD Biosciences, San Diego, CA), IL-1 $\beta$  and IL-6 with the mouse ELISA Ready-SET-Go! kit from eBioscience (Affymetrix, San Diego, CA) and Interferon- $\beta$  was measured with Legend max mouse IFN- $\beta$  ELISA kit (Biolegend). The detection was based on colorimetric quantification of absorbance at 450 nm, corrected with subtraction at 570 nm measured in a microplate reader (Bio-Rad Model 550). NO was estimated from the nitrite concentration measured with a Griess reagent kit at 548 nm (Molecular Probes/Life Technologies, Thermo Fisher Scientific). Results were expressed as the means of duplicate wells.

## Immunoblotting

Total cell extracts from BMDCs stimulated with *Lm*, HKLM or the indicated TLR ligands for the indicated times were prepared in lysis buffer (0.5% Triton X100, 25 mM Tris-HCl pH 7.5, 0.5 mM EGTA, 0.5 mM EDTA, 25 mM NaF, 0.5 sodium glycerol-phosphate, 2.5 mM pyrophosphate, 0.135 M saccharose) with a cocktail of protease and phosphatase inhibitors (Roche). Cell lysates were cleared of nuclei by centrifugation at 15,000 g for 15 min. Protein extracts were separated by 8–15% SDS-PAGE and transferred to a PVDF membrane (Biorad). Proteins were visualized with LAS-3000 after membrane incubation with specific antibodies (see [S1 Table](#)) and peroxidase-conjugated secondary antibodies (5  $\mu\text{g ml}^{-1}$ ). Band intensities were quantified using Image Gauge software (Fuji Photo Film, Co., Ltd) and results are expressed relative to loading controls. For quantification of western-blots, phosphorylated/total ratios were divided by loading control signal. Non-infection (NI) time was considered as 100%, and following times were relativized to it.

## Immunoprecipitation of MyD88 and HDAC6 proteins

Human moDCs ( $1 \times 10^7$  per condition) were lysed (10 mM Tris pH 7.4, 150 mM NaCl, 5% glycerol, 1mM EDTA, 1mM  $\text{MgCl}_2$ , 1mM  $\text{CaCl}_2$ , 1% CHAPS (Sigma) and protease and phosphatase inhibitors (Roche)) for 1 h at 4°C. Lysates were incubated for pre-clearing with pre-washed Protein G Dynabeads (Invitrogen; 50  $\mu\text{l}$  per condition; 2 h, 4°C). Pre-cleared lysates were incubated with 6  $\mu\text{g}$  rabbit anti-MyD88 antibody (Cell Signaling) or 6  $\mu\text{g}$  rabbit anti-HDAC6 antibody (Assay bioTech) per condition O/N at 4°C. Similar  $\mu\text{g}$  of control isotype antibody for rabbit were used. Fifty microlitres of Dynabeads per condition were washed three times in wash buffer (10 mM Tris pH 7.4, 150 mM NaCl, 5% glycerol, 1mM EDTA, 1mM  $\text{MgCl}_2$ , 1mM  $\text{CaCl}_2$ , 0.1% CHAPS) and added to antibody-conjugated lysates for 2 h 4°C. Antibody-conjugated Dynabeads were washed six times with wash buffer and transferred to clean tubes.

HEK293T cells or HEK-Blue hTLR2 ( $1 \times 10^7$  per condition) were lysed (25 mM Tris pH 8, 150 mM NaCl, 0.5% NP-40 and protease and phosphatase inhibitors) and incubated for pre-clearing with pre-washed Protein G Dynabeads (Invitrogen; 50  $\mu\text{l}$  per condition; 3 h, 4°C). Fifty microlitres of Dynabeads per condition were washed three times in wash buffer (25 mM Tris pH 8, 150 mM NaCl, 0.05% NP-40) and re-suspended in 600  $\mu\text{l}$  of wash buffer containing 1–2  $\mu\text{g}$  mouse anti-HA antibody (Roche) per condition and incubated 3 h at 4°C. Similar  $\mu\text{g}$  of control isotype antibody for mouse were used. Pre-cleared lysates were incubated with antibody-conjugated Dynabeads (O/N, 4°C). Antibody-conjugated Dynabeads were washed six times with lysis buffer and transferred to clean tubes. Then, were washed twice with wash buffer. Protein loading buffer was added, samples were boiled at 95°C for 5 min and processed for immunoblotting.



## Flow cytometry

Cells were stained in ice-cold PBS containing FBS (0.5%) and EDTA (5 mM) using appropriate antibody-fluorophore conjugates. Multiparameter analysis was performed on a FACSCANTO II flow cytometer (BD Biosciences) and analysed with FlowJo software (Tree Star). Prior to fixing, cells were resuspended in PBS/0.5% BSA/5 mM EDTA solution containing yellow fluorescent reactive dye to exclude dead cells (Life Technologies). For intracellular staining, cells were fixed and permeabilized using the CytoFix/Cytoperm kit (BD).

## Fluorescence confocal microscopy

For immunofluorescence assays, cells were plated onto slides coated with poly-L-lysine (50  $\mu\text{g ml}^{-1}$ ) and incubated for 1 h at 37°C. Infection experiment were carried out at the indicated times. Cells were then fixed, blocked and stained with the indicated primary antibodies (5  $\mu\text{g ml}^{-1}$ ) followed by alexa488- or Rhodamine Red X-labelled secondary antibodies (5  $\mu\text{g ml}^{-1}$ ). Samples were examined under a Leica SP5 confocal microscope (Leica) fitted with a 63X objective. Images were acquired with sequential xyz acquisition mode scans with laser ranges of 418–473 nm for DAPI, 502–548 nm for Alexa-488, 584–644 nm for Rhodamine X and 737–779 nm for Alexa-647. Z-stacks of 2–5  $\mu\text{m}$  were obtained using a maximum z-step size of 0.3  $\mu\text{m}$ .

## Imaris quantification

Images were processed and assembled using Image J 1.51p (Fiji). Confocal 3D images assembled with Imaris 7.7.2 (Bitplane) using the ImarisCell module. Every cell and its corresponding intracellular bacteria were calculated in each image. Surfaces corresponding to bacteria were used to calculate the maximal fluorescence intensity of the channels to co-localize with bacteria. Two-channel co-localization was quantified in at least 10 images per genotype, corresponding to 10 biological samples.

## In-gel protein digestion

Proteins were in-gel digested using a previously described protocol [69]. Briefly, the coimmunoprecipitate was heated at 95°C for 5 min, after which the magnetic beads were removed using a magnet. The resulting solution was added sample buffer and loaded in 0.5-cm-wide wells of an SDS-PAGE gel. The run was stopped as soon as the front entered into the resolving gel. The protein band was visualized by Coomassie Blue staining, excised, and digested overnight at 37°C with 60 ng/ $\mu\text{l}$  sequencing-grade modified trypsin (Promega) at 10:1 protein:enzyme (w/w) ratio in 50 mM ammonium bicarbonate, pH 8.8, containing 10% acetonitrile. The resulting tryptic peptides were desalted onto C18 OMIX tips (Agilent), dried down and kept at -80°C until further use.

## Mass spectrometry

The resulting peptides were analyzed by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) on an Easy nLC-1000 nano-HPLC apparatus (Thermo Scientific, San Jose, CA, USA) coupled to a hybrid quadrupole-orbitrap mass spectrometer (Q Exactive HF, Thermo Scientific). The dried peptides were taken up in 0.1% (v/v) formic acid and then loaded onto a PepMap100 C18 LC pre-column (75  $\mu\text{m}$  I.D., 2 cm, Thermo Scientific) and eluted on line onto an analytical NanoViper PepMap 100 C18 LC column (75  $\mu\text{m}$  I.D., 50 cm, Thermo Scientific) with a continuous gradient consisting of 8–31% B in 240 min (B = 80% ACN, 0.1% formic acid) at 200 nL/min. Peptides were ionized using a Picotip emitter

nanospray needle (New Objective). Each MS run consisted of enhanced FT-resolution spectra (120,000 resolution) in the 400–1,200  $m/z$  range followed by data-dependent MS/MS spectra of the 20 most intense parent ions acquired along the chromatographic run. The AGC target value in the Orbitrap for the survey scan was set to 1,000,000. Fragmentation in the linear ion trap was performed at 30% normalized collision energy with a target value of 10,000 ions. The full target was set to 30,000, with 1 microscan and 50 ms injection time, and the dynamic exclusion was set to 0.5 min.

## Peptide identification

For peptide identification the MS/MS spectra were searched with the Sequest algorithm implemented in Proteome Discoverer 1.4 (Thermo Scientific). Database searching against human protein sequences from the UniProt database (March 2017, 158,382 entries) was performed with the following parameters: trypsin digestion with 4 maximum missed cleavage sites; precursor and fragment mass tolerances of 800 ppm and 0.02 Da, respectively; Cys carbamidomethylation as static modifications; and Met oxidation and Lys acetylation as dynamic modifications. The results were analyzed using the probability ratio method [70] and a false discovery rate (FDR) for peptide identification was calculated based on the search results against a decoy database using the refined method [71].

## Statistical analysis

Data were analysed with GraphPad prism software (La Jolla, CA) for normality (Kolmogorov-Smirnov test for small samples). Normal data were analysed by Student t-test, non-normal data by Mann-Whitney test, and grouped data by 2-tailed One-way ANOVA with a Bonferroni post-test. For western blot quantification, the sample with the maximum signal was assigned a value of 100%, and signals in other samples were expressed as a percentage of this; significance was determined by a one-sample test. Long-rank (Mantel-Cox) test and Cehan-Breslow-Wilcoxon test were used for the analysis of the Kaplan-Meier curve (survival curve).

## Supporting information

**S1 Fig. Differentiation of GM-CSF-derived DCs, their viability at 6 hpi, comparison of CFUs of GM-CSF- and M-CSF-derived cells and fluorescent confocal microscopy of *Lm*.**  
A) Left: Dot-plots showing CD11c and MHC-II markers, with gating for CD11c<sup>+</sup>MHC-II<sup>+</sup> and CD11c<sup>+</sup>MHC-II<sup>-</sup> populations (percentages indicated). Right: Dot-plots on differentiation day 11 showing FSC-H versus Gr-1, gating the Gr-1<sup>+</sup> population corresponding to neutrophil contamination in GM-CSF-derived DC cultures. Charts show the percentages of CD11c<sup>+</sup>MHC-II<sup>+</sup>, CD11c<sup>+</sup>MHC-II<sup>-</sup> and Gr-1<sup>+</sup> populations. ns>0.05 non-significant; n = 6.  
B) Percentage viability of BMDCs before infections and at 6 hpi with *Lm*, ns>0.05 non-significant; n = 6.  
C) Comparison of CFUs in GM-CSF-derived DCs and M-CSF-derived macrophages over the time-course of *Lm* infection. \*\*\*p≤0.001, \*\*p≤0.01, ns>0.05 non-significant; n = 6.  
D) ImarisCell Module analysis of the number of cells and the number of bacteria per cell in all pictures (10 pictures per genotype). The graph shows the distribution of cells with a specific number of bacteria per cell. The number of cells with 6 and 7 bacteria differed significantly between the *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> genotypes. \*p≤0.05, n = 10.  
E) Confocal microscopy determination of bacterial load of the Fig 1F. Maximum intensity z-projections of confocal microscopy images of *Lm*-infected *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> BMDCs at 6 hpi. ImarisCell Module view of the number of nucleus and bacteria per cell. Actin transparency is used to visualize bacteria (number indicated on the right). Images show DAPI (blue),

*Lm* (red),  $\beta$ -actin (green). Scale bars 20  $\mu$ m.  
(TIF)

**S2 Fig. Gating strategy of myeloid populations of the spleen.** A) Dot-plots showing the gating of myeloid populations of spleen. Dot-plots showing SSC-A versus FSC-A indicates p1, FSC-H versus FSC-W and SSC-H versus SSC-W were used to avoid doublets and FSC-H versus viability shows live and dead cells. Singlets and live cells were used to choose CD3<sup>+</sup>CD19<sup>+</sup>DX5<sup>+</sup>Ly6G<sup>+</sup> cell population. From this population, neutrophils were gated as Ly6G<sup>+</sup>Ly6C<sup>+</sup> cells, monocytes as CD11b<sup>+</sup>CD11c<sup>lo</sup>, Tips DCs as intermediate levels of CD11b and CD11c, conventional dendritic cells (cDCs) as CD11c<sup>hi</sup>; inside this population cDCs CD8<sup>+</sup> were distinguished as CD11c<sup>hi</sup>CD11b<sup>+</sup> and cDCs CD8<sup>+</sup> as CD11c<sup>hi</sup>CD11b<sup>lo</sup>. B) Representative histograms of different splenic populations (monocytes, neutrophils, Tips DCs, total cDCs, cDCs CD8<sup>+</sup> and cDCs CD8<sup>+</sup>) show *Lm* signal of *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> mice injected with a lethal dose of *Lm* at 6 hpi. A pool of *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> spleens non-infected was used as a control sample without infection (NI). \*\* $p \leq 0.01$ , \* $p \leq 0.05$ ; n = 6.  
(TIF)

**S3 Fig. Control vehicles and autophagy markers.** A) Total CFUs at 0 and 6 hpi in *Lm*-infected BMDCs (MOI of 10) treated with different control vehicles (H<sub>2</sub>O, DMSO and ethanol). H<sub>2</sub>O were the control vehicle used for NH<sub>4</sub>Cl and cloroquine, DMSO for 3-MA, bafilomycin A1, DPI and 1400W and ethanol for rapamycin. Time 0 is included as a bacterial entry control. \*\*\* $p \leq 0.001$ , ns>0.05 non-significant; n = 6. B) PCR analysis of autophagy markers (ATG-2, 5, 7 and 12, LC3A and B, p62 and Beclin-1) and lysosome markers (LAMP-1 and 2) (arbitrary units) after 6 hpi with *Lm*, ns>0.05 non-significant; n = 5.  
(TIF)

**S4 Fig. Pro-inflammatory cytokine secretion.** ELISA detection of the pro-inflammatory cytokines IL-1 $\beta$  and IL12p70 (pg/ml) in supernatants (S) and in supernatants plus the corresponding cell pellets (S+P) of *Lm*-infected *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> BMDCs at 6, 12 and 24 hpi. \*\*\* $p \leq 0.001$ , \*\* $p \leq 0.01$ , \* $p \leq 0.05$ , ns>0.05 non-significant; n = 5.  
(TIF)

**S5 Fig. TLR expression and TLR-signalling pathway activation by LPS and HKLM.** A) Western-blot analysis in *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> BMDCs over the time-course of LPS or HKLM treatment. Total and phosphorylated AKT were detected for both treatments. Accompanying charts on the right show quantification, indicating the percentage of pAKT/total AKT ratio. \*\* $p \leq 0.01$ , \* $p \leq 0.05$ ; n = 4. B) PCR analysis of TLR-1, 2 and 6 (arbitrary units) in *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> BMDCs non-infected (NI) and after *Lm*-infection at 6 hpi. ns>0.05 non-significant; n = 6.  
(TIF)

**S6 Fig. Differentiation of FLT3-L DCs, their pro-inflammatory cytokine secretion at 6 hpi and association of HDAC6 with TLR-adaptor MyD88.** A) Left: Dot-plots of FLT3-L DC cultures at day 11 of differentiation, showing gating for the CD11c<sup>+</sup> population (percentages indicated). Centre: Dot-plots showing CD11b versus B220 to select two populations: CD11c<sup>+</sup>CD11b<sup>+</sup>B220<sup>+</sup> (plasmacytoid DCs, pDCs) and CD11c<sup>+</sup>CD11b<sup>+</sup>B220<sup>-</sup> (conventional DCs, cDCs) (percentages indicated). Right: Dot-plots showing CD11b versus CD24 to select the CD11b<sup>+</sup>CD24<sup>+</sup> and CD11b<sup>+</sup>CD24<sup>-</sup> populations (gated from cDCs) (percentages indicated). The charts on the right show the percentages of CD11c<sup>+</sup>, CD11c<sup>+</sup>CD11b<sup>+</sup>B220<sup>-</sup>CD24<sup>-</sup> and CD11c<sup>+</sup>CD11b<sup>+</sup>B220<sup>-</sup>CD24<sup>+</sup> populations, ns>0.05 non-significant; n = 6. B) ELISA detection of the pro-inflammatory cytokines IL-1 $\beta$  and IL-6 (pg/ml) in supernatants of



*Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> FLT3L-DCs activated with LPS, Imiquimod, Pam3GSK4, HKLM, HKST, *Lm*, Poly(I:C) or flagellin for 6 h. \*\*\**p*≤0.001, \*\**p*≤0.01, \**p*≤0.05; *n* = 6. C) MyD88 adaptor protein in *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> BMDCs. Western-blot analysis of MyD88 over the time-course of *Lm* infection in *Hdac6*<sup>+/+</sup> and *Hdac6*<sup>-/-</sup> BMDCs (left). Accompanying charts on the right show quantification of the percentage of MyD88; ns non-significant; *n* = 5. D) Immunoprecipitation of HA (MyD88) followed by western-blot for HDAC6 and MyD88. Immunoprecipitations were carried out using different HDAC6-eGFP plasmids co-transfected with MyD88-HA in HEK cell line. Over-expressed (HDAC6-eGFP, 160 kDa) is indicated at right of western-blot. E) Immunoprecipitation of HA (MyD88) followed by mass spectrometry analysis. Immunoprecipitations were carried out using different HDAC6-eGFP plasmids co-transfected with MyD88-HA in HEK cell line. The number of unique MyD88 and HDAC6 peptides identified is indicated. (\*) indicates the presence of acetylated MyD88 peptides. Similar results were obtained in three independent experiments. F) MS<sup>2</sup> fragmentation spectra from the peptides showing at 1217.0699 (Top), and 599.3803 (Bottom). Ion adscription to carboxy- (*y* ions, blue) and amino-terminal (*b* ions, red) fragmentation series is indicated. *K*<sub>ac</sub> denotes acetylated lysine and *C*<sub>cm</sub> indicates carbamidomethylated cysteine. Fragment ion sequence coverage is schematically indicated. Similar results were obtained in three independent experiments. (TIF)

**S1 Table. Antibody table.** Table of antibodies used in experimental procedures disclosed by reference, brand, host, application and dilution.

(PDF)

**S2 Table. qPCR primers.** Table of qPCR primers used in experimental procedures disclosed by gene name and sequence 5' -3'.

(PDF)

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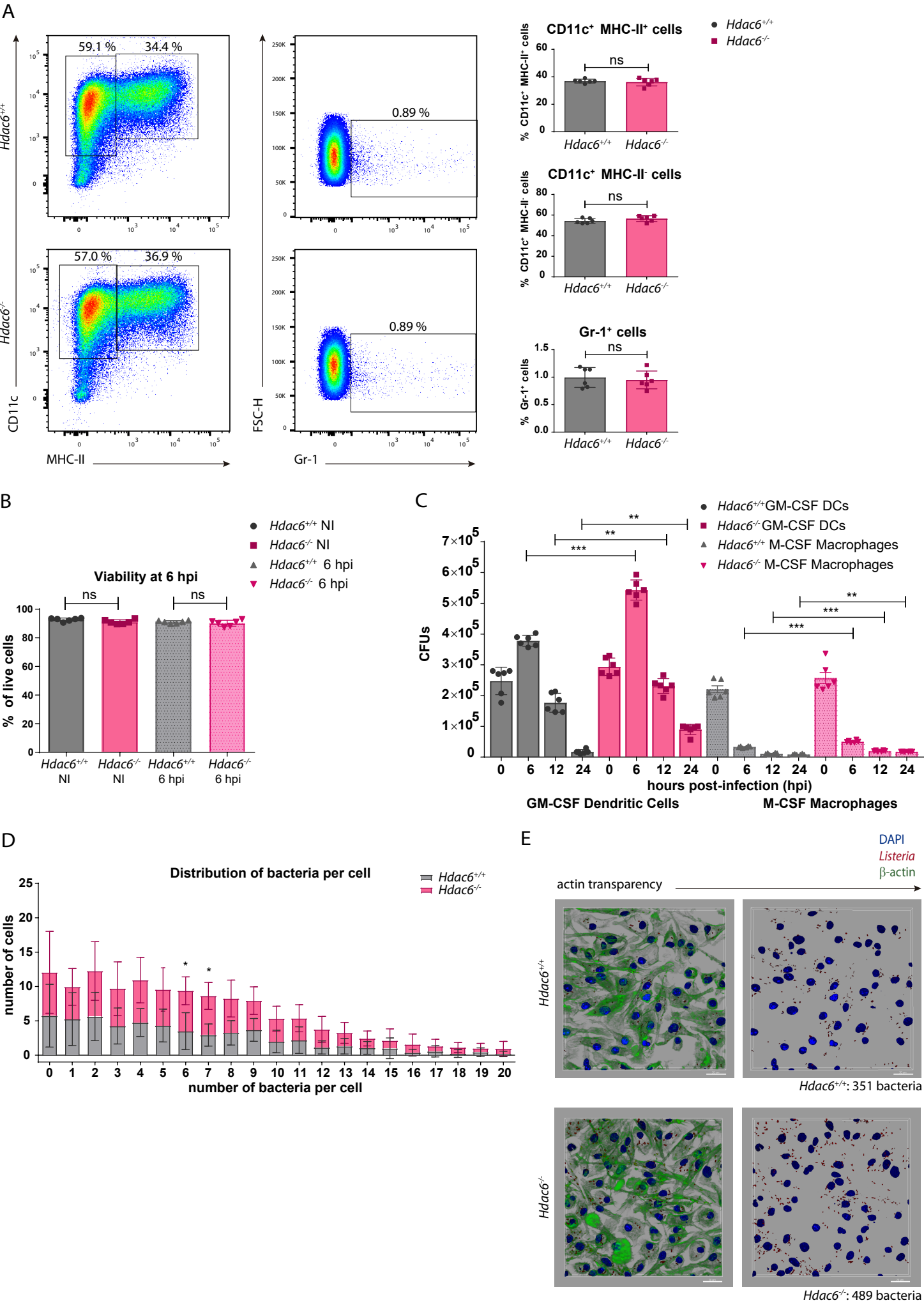
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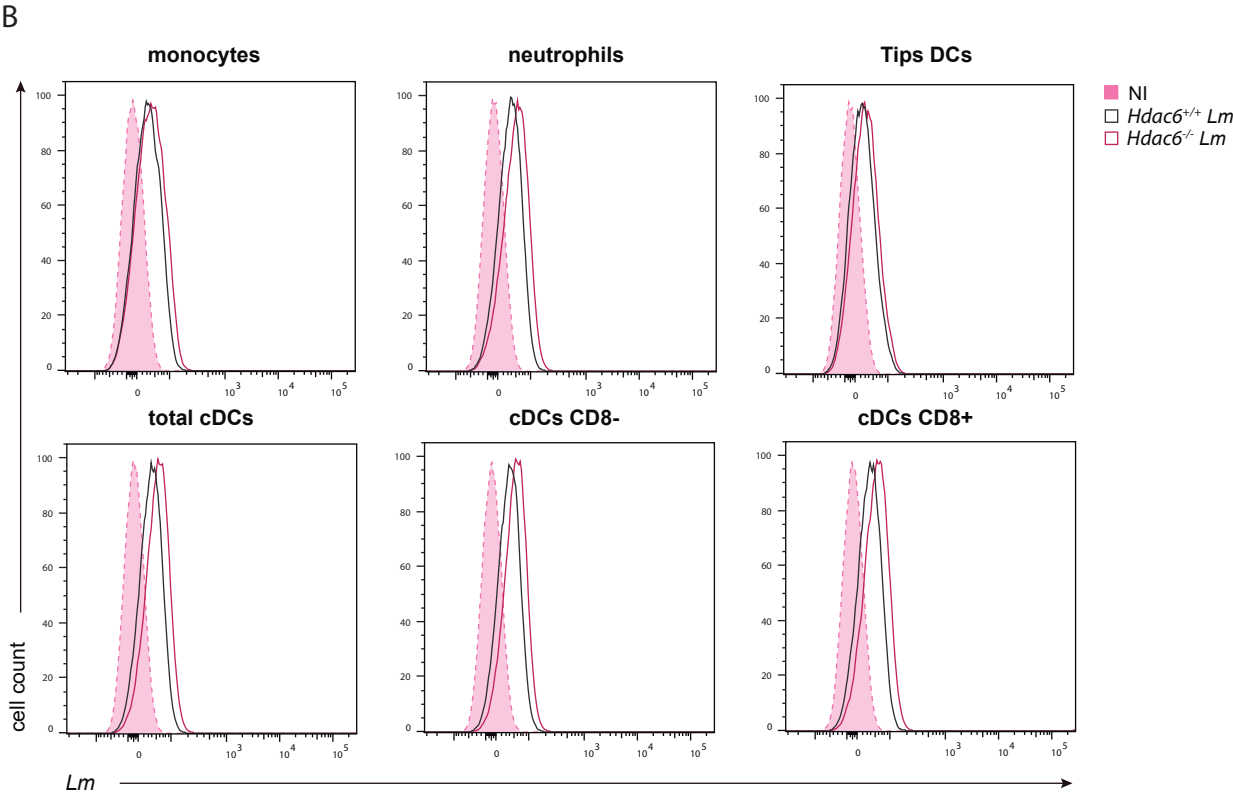
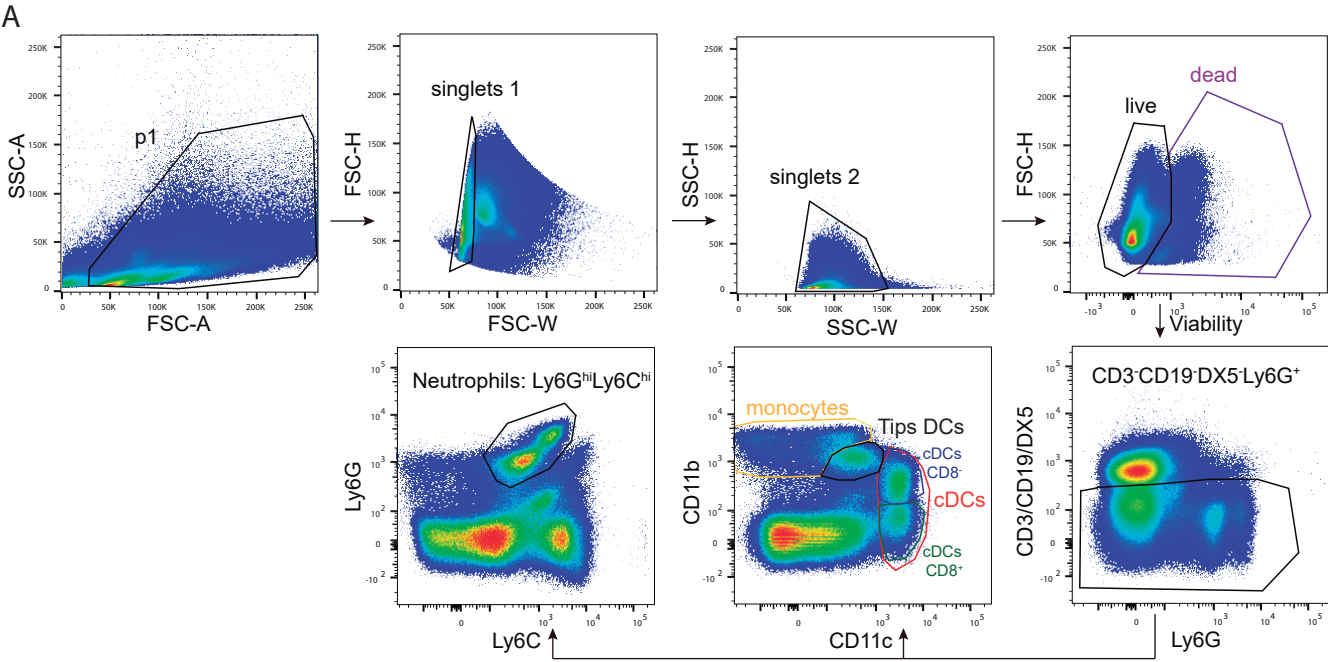
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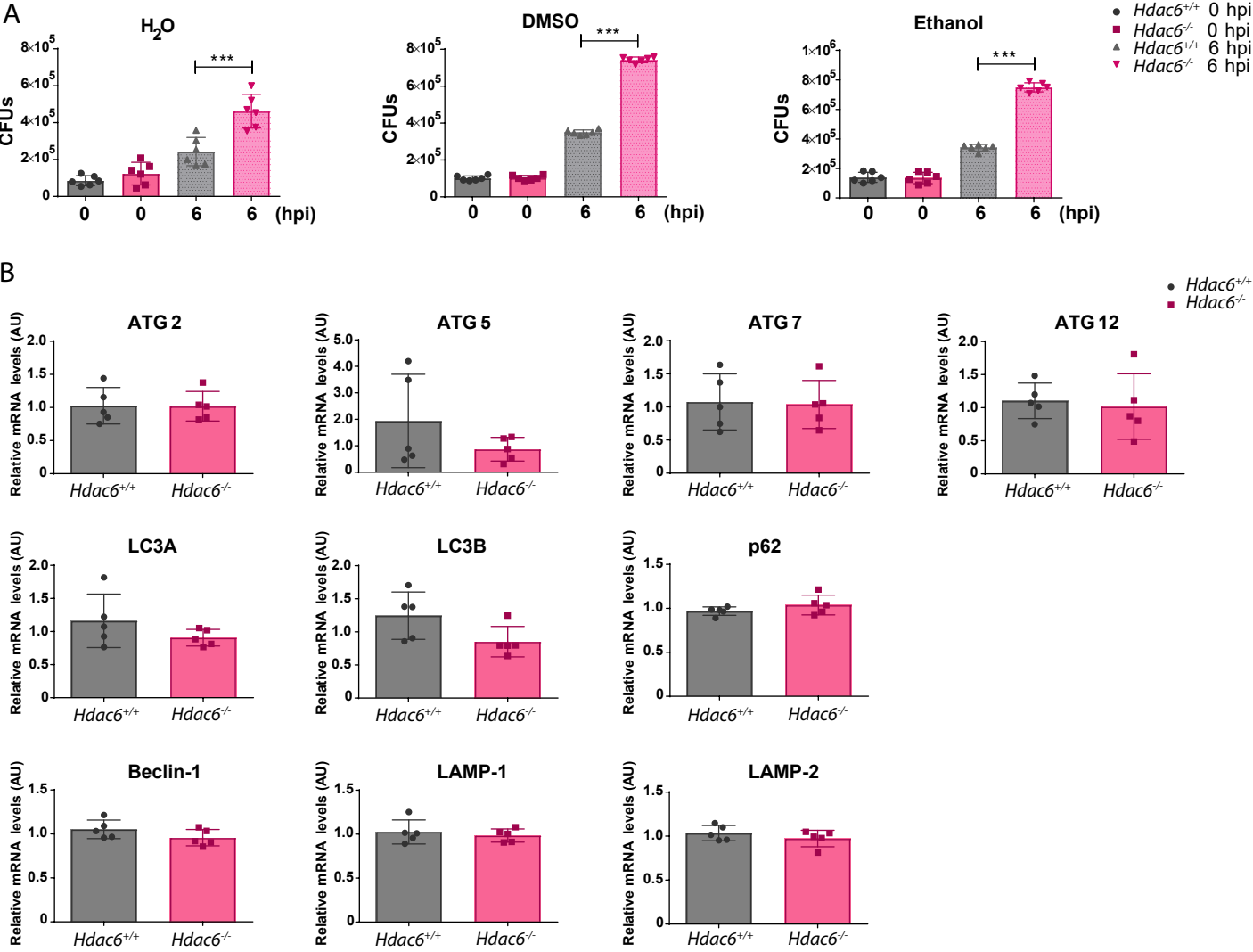
Supplemental Figure 1



Supplemental Figure 2

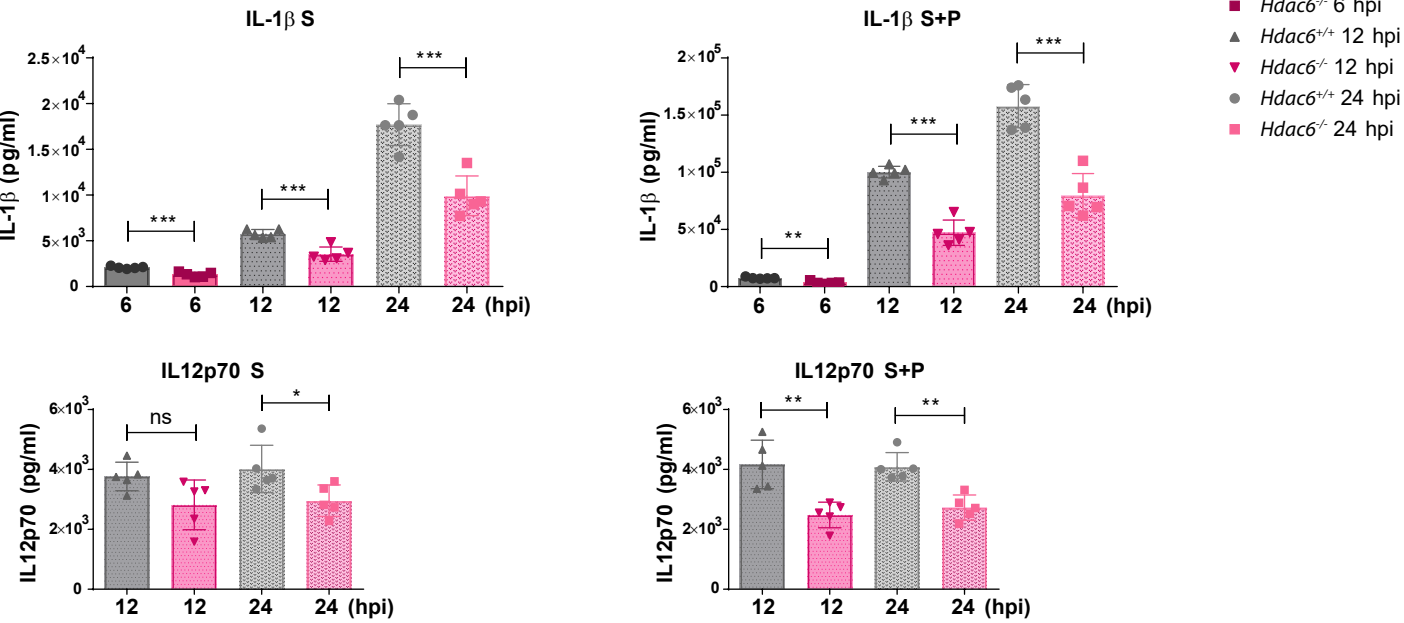


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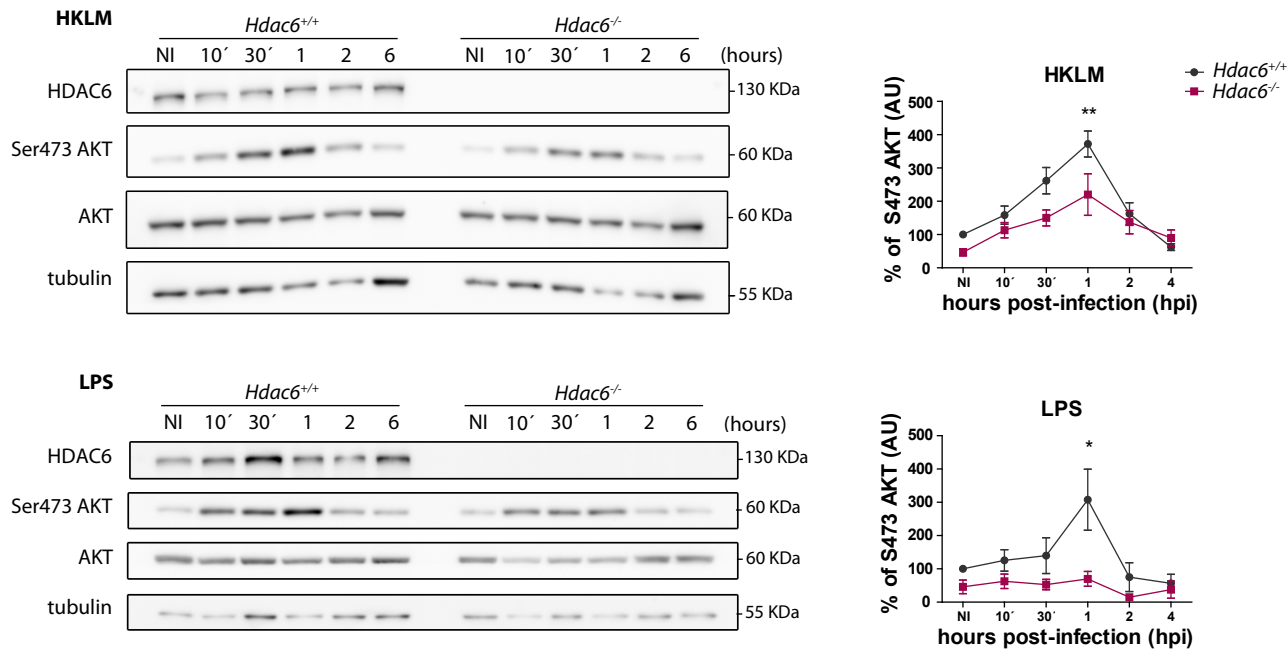


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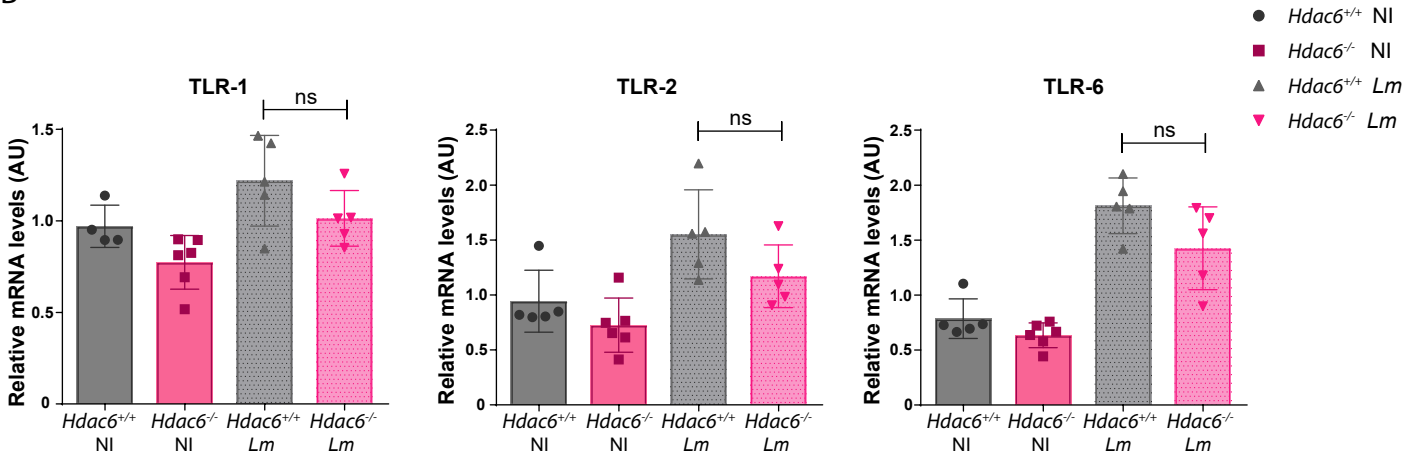


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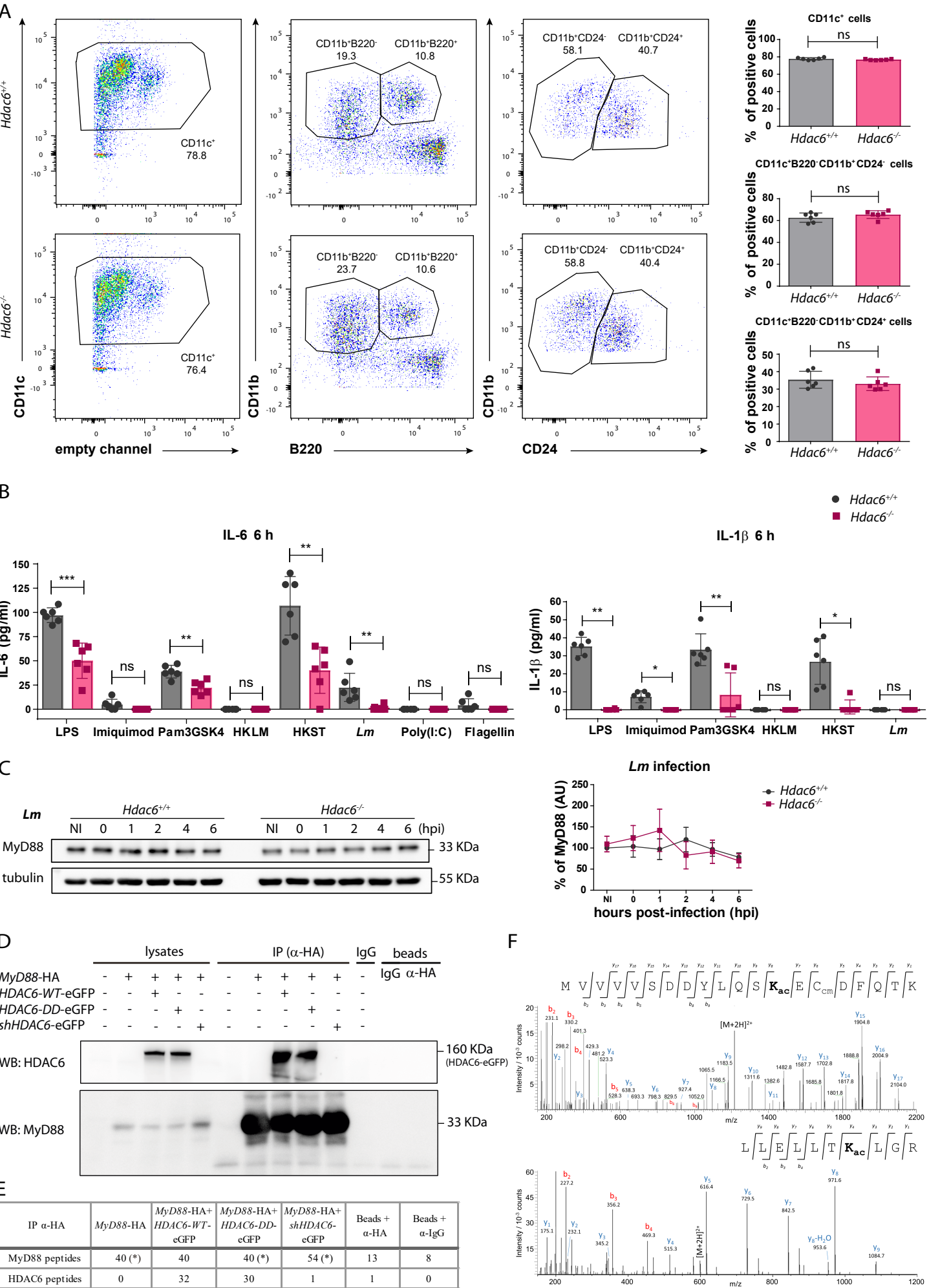
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Supplemental Figure 6



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## HDAC6 regulates the dynamics of lytic granules in cytotoxic T lymphocytes

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### Abstract

HDAC6 is a tubulin deacetylase involved in many cellular functions related to cytoskeleton dynamics including cell migration and autophagy. In addition, HDAC6 affects antigen-dependent CD4<sup>+</sup> T cell activation. In this study, we show that HDAC6 contributes to the cytotoxic function of CD8<sup>+</sup> T cells. Immunization studies revealed defective cytotoxic activity *in vivo* in the absence of HDAC6. Adoptive transfer of wild-type or *Hdac6*<sup>-/-</sup> CD8<sup>+</sup> T cells to *Rag1*<sup>-/-</sup> mice demonstrated specific impairment in CD8<sup>+</sup> T cell responses against vaccinia infection. Mechanistically, HDAC6-deficient cytotoxic T lymphocytes (CTLs) showed defective *in vitro* cytolytic activity related to altered dynamics of lytic granules, inhibited kinesin 1 – dynactin mediated terminal transport of lytic granules to the immune synapse and deficient exocytosis, but not to target cell recognition, T cell receptor (TCR) activation or interferon (IFN $\gamma$ ) production. Our results establish

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**Author contribution.** NNA, NBMC and FSM designed experiments, made the figures and wrote the manuscript; NNA, SI, NBMC, OMG, JV, DS, GM and TSY and EC collected and/or analyzed data; AT developed the Quant Application.

**Conflict of interest.** Authors declare that they have no conflict of interest.

HDAC6 as an effector of the immune cytotoxic response that acts by affecting the dynamics, transport and secretion of lytic granules by CTLs.

## Introduction

Cytotoxic T Lymphocytes (CTLs) are a specialized population of CD8<sup>+</sup> T cells that provides defense against virus-infected cells and tumors. Naïve CD8<sup>+</sup> T cells differentiate into CTLs upon antigen recognition, a process involving the synthesis and storage of cytotoxic mediators into lysosomal-derived lytic granules (LG) (Williams and Bevan, 2007). CTLs eliminate target cells by different mechanisms, including secretion of pro-inflammatory cytokines, e.g. tumor necrosis factor (TNF) $\alpha$  or interferon (IFN) $\gamma$  (de Saint Basile et al., 2010), FAS-L (FAS ligand) ligation to its receptor as well as granule-mediated apoptosis upon cell-cell contact and immune synapse (IS) formation (Ritter et al., 2013). LG fuse with the plasma membrane and release granzymes, cathepsins and perforins (Prf) (Lopez et al., 2013; Pardo et al., 2009). The IS acts as a focal point for exocytosis of LG. LG polarization towards the target cell depends on T cell receptor (TCR) engagement, driven by the relocation of the centrosome to IS. The LG degranulate at a secretory domain adjacent to the TCR-enriched region within the IS (de Saint Basile et al., 2010; Ritter et al., 2013).

Histone deacetylase 6 (HDAC6) is an ubiquitous, cytosolic protein from the class II HDACs family with X-linked inheritance, that binds to and deacetylates  $\alpha$ -tubulin at lys40 (Hubbert et al., 2002; Valenzuela-Fernandez et al., 2008). HDAC6 also modulates other substrates, e.g. cortactin or hsp90. HDAC6 controls cell migration (Zhang et al., 2007), T-regulatory functions (de Zoeten et al., 2011) and CD4<sup>+</sup> T cell activation (Serrador et al., 2004). Consistent with this, HDACs inhibitors impair some immune functions (Mosley et al., 2006; Tsuji et al., 2015). However, the precise contribution *in vivo* (by using *Hdac6*<sup>-/-</sup> mice) has not been assessed, and the mechanisms involved remain unsolved. HDAC6 also functions as a scaffold protein in T cell migration (Cabrero et al., 2006) and the transport of misfolded proteins (Kawaguchi et al., 2003). In this report, we describe the impaired killing capacity of *Hdac6*<sup>-/-</sup> CTLs. The molecular mechanism underlying this defect involves a scaffold role that positions HDAC6 as a protein that oversees the proper movement of LG, their transport to the IS and secretion towards the target cell.

## Results and Discussion

### HDAC6 deficiency reduces the cytolytic capacity of CD8<sup>+</sup> T lymphocytes

We examined the ability of cytotoxic T cells from *Hdac6*<sup>-/-</sup> mice to kill target cells *in vitro*. CD8<sup>+</sup> T cells from wild-type (WT) and *Hdac6*<sup>-/-</sup> mice expressing the transgenic ovalbumin (OVA)-specific TCR (OT-I) were activated *in vitro* and cultured to generate CTLs. Cell cytotoxicity was subsequently analyzed by survival of dye-labeled EL4 target cells pulsed or not with OVA<sub>257-264</sub> peptide (SIINFEKL). *Hdac6*<sup>-/-</sup> CTLs showed decreased killing activity (Fig. 1A), consistent with reduced expression of CD107a (also known as Lamp1) in *Hdac6*<sup>-/-</sup> CTLs upon degranulation (Fig. 1B). Likewise, CTLs from OT-I mice treated with tubastatin A, a potent HDAC6 inhibitor, displayed a reduced killing ability (Suppl. Fig. 1A). We also detected decreased Prf1 secretion from activated (i.e. induced by anti-CD3 and anti-

CD28 monoclonal antibodies) *Hdac6*<sup>-/-</sup> CTLs (Fig. 1C, left). Next, we assessed the secretion promoted by phorbol-12-myristate-13-acetate (PMA), to bypass TCR stimulation. Both cathepsins D and Prf1 decreased in supernatants from activated *Hdac6*<sup>-/-</sup> CTLs (Fig. 1C, right). Taken together, our data demonstrate that *Hdac6*<sup>-/-</sup> CTLs show reduced cytotoxic activity, and suggest that HDAC6 controls exocytosis.

We next tested IFN- $\gamma$  production; the frequency of CTLs producing IFN $\gamma$  and its secretion was unaffected in activated *Hdac6*<sup>-/-</sup> CTLs (Fig. 1D-E), in contrast to what has been described when cells are treated with ACY-1215, a recently described inhibitor that is ten-fold more selective for HDAC6 than for HDAC1, HDAC2 and HDAC3 and that shows slight activity against HDAC8 (Tsuji et al., 2015). Likewise, treatment of CTLs from OT-I mice with tubastatin A had no significant effect (Suppl. Fig. 1B). Importantly, T-cell signaling induced by anti-CD3/anti-CD28 mAbs in *Hdac6*<sup>-/-</sup> was comparable to control, as determined by assessing PLC $\gamma$ 1 and erk1/2 (Erk1/2; also known as MAPK3 and MAPK1, respectively) phosphorylation (Fig. 1F). Likewise, the increase in intracellular calcium remained unchanged upon activation (Fig. 1G). As expected, tubulin acetylation at Lys40 was increased in *Hdac6*<sup>-/-</sup> CTLs (Fig. 1F). These results suggest that the killing defect observed does not result from a general impairment of CTLs function.

### Defective *in vivo* and *ex vivo* killing in HDAC6 knockout mice

The effector activity of *Hdac6*<sup>-/-</sup> CD8<sup>+</sup> T cells was tested *in vivo* following mouse immunization using SIINFEKL-pulsed dendritic cells. The *in vivo* killing activity against the injected target cells (pulsed or not with SIINFEKL) was analyzed upon recovery by peritoneal lavage. Notably, *Hdac6*<sup>-/-</sup> mice showed reduced specific killing of target cells (Fig. 2A, left panel). However, the proportion of SIINFEKL-specific CD8<sup>+</sup> T cells from the endogenous repertoire was not affected in *Hdac6*<sup>-/-</sup> mice (Fig. 2A, right panel), suggesting that the cytotoxic function rather than the number of antigen-specific CTLs could underlie the defect. Next, we examined whether the decreased cytotoxic function of the CTLs resulted in an impaired ability to prevent morbidity and/or mortality during a viral infection. To restrict HDAC6 deficiency to CD8<sup>+</sup> T cells, we adoptively transferred *Rag1*<sup>-/-</sup> mice with WT or *Hdac6*<sup>-/-</sup> naïve CD8<sup>+</sup> T cells and subsequently challenged with a fully replicative vaccinia virus (VACV) WR strain. This infection model mimics the immunological and clinical features of smallpox vaccination in humans (Mota et al., 2011). CD8<sup>+</sup> T cell proliferation was comparable, or even increased (division 4) in *Hdac6*<sup>-/-</sup> (Fig. 2B). *Rag1*<sup>-/-</sup> mice passively transferred with *Hdac6*<sup>-/-</sup> CD8<sup>+</sup> T cells showed increased morbidity at 9 and 11 days post-infection (p.i.) (Fig. 2C). Virus titration from the lesion tissue demonstrated that *Hdac6*<sup>-/-</sup> immune cells exerted a less-efficient virus clearance (Fig. 2D). Consistent with our findings on the lack of effect in endogenous antigen-specific CD8<sup>+</sup> T cell numbers, CTL expansion tracked at 13 d.p.i. was not affected in *Hdac6*<sup>-/-</sup> (Fig. 2E, left). The proportion of activated CD8<sup>+</sup> T cells (CD44<sup>high</sup>) at early (5 d.p.i.) and late stages (13-30 d.p.i.) of the disease were similar for WT and *Hdac6*<sup>-/-</sup> mice (tested in peripheral blood and spleen, respectively; Fig. 2E, right). These *in vivo* results emphasize the role of HDAC6 in the CD8<sup>+</sup> T cell-dependent protection against VACV infection without affecting effector CD8<sup>+</sup> CTLs differentiation.



## HDAC6 drives the terminal transport of LG to the target cell

CTL killing is limited to target cells (and not neighbor cells) by the confinement of secretion to the immune synapse established between the CTL and the target cell (de Saint Basile et al., 2010). Interestingly, the intracellular colocalization between cathepsinD and lamp1 (CD107a) was affected in *Hdac6*<sup>-/-</sup> CTLs conjugated with target cells, pointing to the mislocalization of lytic mediators in these cells (Fig. 3A; images and middle graphs). The decreased secretion of lytic proteins from *Hdac6*<sup>-/-</sup> CTLs suggests that HDAC6 regulates exocytosis of LG (Fig. 1C). Indeed, the translocation of the centrosome to the contact area with the target cell was even more pronounced in *Hdac6*<sup>-/-</sup> CTLs than in WT cells (Fig. 3A, right graph), in accordance with the effect described with the HDAC inhibitor Trichostatin A on the centrosomal polarization in CD4<sup>+</sup> T cells (Serrador et al., 2004). This suggests that the defective exocytosis might rely on the movement of LG themselves.

We thus monitored the dynamics of LG at the subcortical immune synapse cytoskeleton and their release by Total Internal Reflection Fluorescence microscopy (TIRFm). CTLs were loaded with a pH-dependent, lysosomal tracker which allows the visualization of the LG and settled on to a stimulating surface to form an IS-like structure (Fig. 3B). These experiments revealed significant changes in the distribution of the LG and their dynamics, with a marked decrease in the number of LG detected at the immune synapse-like structure in *Hdac6*<sup>-/-</sup>, suggesting alterations to the active transport of the granules from the centrosomal region to the plasma membrane. Indeed, the mean fluorescence intensity detected for *Hdac6*<sup>-/-</sup> granules was lower, which suggests a higher pH and therefore a different degree of maturation, although the LG displayed similar sizes in WT and *Hdac6*<sup>-/-</sup> cells (Fig. 3C). The most remarkable difference pertained to the *x-y* distribution of the LG, which was wider (diffusion surface) in the *Hdac6*<sup>-/-</sup> CTLs, with a higher diffusion coefficient, although maintaining similar duration times and path lengths (Fig. 3D). These data suggest that the LG from *Hdac6*<sup>-/-</sup> CTLs are not properly targeted and/or that they dock inefficiently at the immune synapse.

Tubulin motors control the delivery of LG to the plasma membrane. Whereas dynein controls LG targeting to the centrosome (Burkhardt et al., 1993; Mentlik et al., 2010), the kinesin-1/Slp3/Rab27a (Slp3 is also known as SYTL3) complex directs terminal transport to the plasma membrane for exocytosis (Kurowska et al., 2012). Dynactin might also be part of this complex, linking the cargo to kinesin-1 motor (Haghnia et al., 2007; Hendricks et al., 2010). We then hypothesized that HDAC6 regulates the movement and delivery of the LG at the IS through kinesin-1. Using a biochemical approach, we observed that HDAC6 formed a complex with kinesin-1 light chain (KLC1) upon triggering with anti-CD3 and anti-CD28 monoclonal antibodies (Fig. 3E). Moreover, interaction of the kinesin-activator complex dynactin subunits p150-glued (also known as DCTN1) and p50-dynamitin (also known as DCTN2) was impaired in *Hdac6*<sup>-/-</sup> (Fig. 3F).

In summary, our data support a specific role for HDAC6 in the intracellular localization of lytic mediators and, particularly, in their exocytosis. Therefore, the catalytic and scaffold activities of HDAC6 might act at multiple levels in the control of cytotoxic-related pathways, making HDAC6 a potential candidate that could be targeted to modulate CTLs in specific diseases.

## Materials and Methods

### Mice

*Hdac6*<sup>-/-</sup> mice were generated through targeting of exons 10 to 13 (Gao et al., 2007). They were intercrossed in a C57BL/6 background to generate wild-type and knockout littermates. Mice presenting transgenic inserts for mouse *Tcra*-Variable 2 and *Tcrb*-Variable 5 genes, which TCR recognizes ovalbumin<sub>257-264</sub> peptide in the context of H2Kb MHC-I (OT-I), were crossed to female *Hdac6*<sup>+/-</sup> mice to generate WT and KO littermates; males were used for *in vivo* experimentation since *Hdac6* is a X-linked gene. *Rag1*<sup>-/-</sup> mice were used for adoptive transfer experiments. These studies were approved by the local Ethics Committee for Basic Research at the CNIC and the Comunidad Autónoma de Madrid.

### Cell culture

Cytotoxic cells were produced by culturing cells upon stimulation with SIINFEKL peptide (0.5  $\mu$ M, 24h) or concanavalin A (2.5  $\mu$ g/ml, 36 h) and cultured in presence of IL-2 (50-100 IU/ml) for at least 7 days. All other cells were cultured and treated as described previously (Cascio et al., 2015; Martin-Cofreces et al., 2006; Sancho et al., 2008).

### Immunoprecipitation, CTL signaling and immunoblotting

Experimentation was performed as described (Martin-Cofreces et al., 2012; Martin-Cofreces et al., 2008; Martin-Cofreces et al., 2006). ); KLC1 antibody was from Merck Millipore (5  $\mu$ g/ml for Immunoprecipitation and 1  $\mu$ g/ml for Western blot; Darmstadt, Germany; KLC), anti-HDAC6 from Assay Biotech (0.1  $\mu$ g/ml for Western blot; Sunnyvale, California, US) and anti-p50 (0.25  $\mu$ g/ml for Western blot) and -p150 (1  $\mu$ g/ml for Immunoprecipitation and 0.25  $\mu$ g/ml) for Western blot from BD Pharmingen (Franklin Lakes, New Jersey, US).

### Measurement of intracellular variations in Calcium ions by flow cytometry

The method used for intracellular calcium influx is described (June and Moore, 2004). In particular,  $5 \times 10^6$  purified CD8<sup>+</sup> CTLs generated *in vitro* were loaded with 2  $\mu$ g.ml<sup>-1</sup> INDO-1 AM (Invitrogen Corporation) and stimulated with anti-CD3 and anti-CD28 monoclonal antibodies (BD Biosciences; Franklin Lakes, New Jersey, US) plus goat anti-Armenian-hamster IgG antibodies (Jackson ImmunoResearch Laboratories; West Grove, PA, US. 6, 3 and 6  $\mu$ g/ml, respectively).

### In vitro degranulation assay

CD107a expression was monitored with anti-CD107a-Alexa647 antibody (BD Biosciences) in monensin-pretreated CD8<sup>+</sup> OTI cells (5 mM) stimulated with SIINFEKL-pulsed EL4 (1  $\mu$ M; 3 h, 37 °C). Cells were stained with anti-CD8-PE and anti-CD44-FITC, analyzed by FACS and data were processed with FlowJo 7.6.5 (TreeStar Inc; Ashland, Oregon; US).

### Confocal and Total Internal Reflection Fluorescence Microscopy analysis

Cell conjugates between CTLs and EL4 cells were allowed to form (15 min) and processed as described (Cascio et al., 2015; Martin-Cofreces et al., 2006) under a Leica SP5 confocal microscope (Leica Microsystems; Mannheim, Germany) mounted on an inverted DMI6000



microscope fitted with a HCX PL APO 63x/1.40-0.6 oil objective. Images were processed using Imaris software (Bitplane; Zurich, Switzerland), Image J software (<http://rsbweb.nih.gov/ij/>) and assembled with Photoshop 6 software. 3D distance from the centrosomal centre of mass to the target cell-edge was measured by generating image masks from fluorescence with Imaris Software. TIRFm imaging was performed with a Leica AM-TIRF-MC-M system mounted on a Leica DMI-6000B microscope coupled to an Andor-DU8285\_VP-4094 camera (Andor; Belfast, UK) fitted with a HCX-PL-APO 100.0x1.46 OIL objective as described (Baixauli et al., 2011; Martin-Cofreces et al., 2012). The laser penetrance used was 90 nm (561 nm laser). The LG mechanical properties were determined with a user-customized routine developed in Python. The software can be freely downloaded from: <https://dl.dropboxusercontent.com/u/4050954/VesiclesAnalyser.zip>. For more information, see the tutorial included.

### **Vaccinia virus (VACV) infection and virus titration**

Tails were scarified with VACV ( $2 \times 10^6$  PFU/mouse) by gently scratching (x25) with a 28 1/2 G needle. For virus titration, tails were mechanically disaggregated (1ml of PBS), subjected to freeze-thaw cycles and sonication. Serial dilutions of the homogenates were added to monolayers of CV-1 cells seeded in 24 well plates. Cells were stained with Cristal violet 24 h later. We observed a detection limit of 5 PFU/tail, the number of plaques was multiplied by the reciprocal of sample dilution and converted to p.f.u./g of tissue.

### ***In vitro* and *in vivo* cytotoxicity assay**

For *in vitro* experiments, EL4 target cells were incubated with 1  $\mu$ M Cell Violet pulsed with 1  $\mu$ M SIINFEKL or with 0.1  $\mu$ M Cell Violet and no SIINFEKL, washed extensively, mixed (1:1), pooled with different dilutions of effectors, plated in a 96-well U-bottom plate for 5 h (37°C) by triplicate and analyzed by FACS. Dead cells were excluded on the basis of propidium iodide staining. The mean percentage of survival in antigen-loaded targets was calculated relative to antigen-negative internal controls in each sample. Specific lysis was calculated using the following equation: percentage specific lysis =  $100 * (1 - (\text{percentage of cells staining for Cell Violet } 1\mu\text{M} / \text{percentage of cells staining for Cell Violet } 0,1\mu\text{M}))$ . All data were normalized to the basal specific lysis in absence of effector cells. For *in vivo* assays, WT and *Hdac6*<sup>-/-</sup> mice were immunized by i.p. injection of bone marrow dendritic cells pulsed with 1  $\mu$ M of SIINFEKL and LPS ( $1 \mu\text{gml}^{-1}$ ) for 1 h. After 7 days, CD45.1 splenocytes were prepared as targets as described above and injected i.p. into recipients. Cells were recovered 24 h later by peritoneal lavage and *in vivo* killing measured (Hermans et al., 2004; Iborra et al., 2012; Sancho et al., 2008; Schulz et al., 2005).

### **Statistical analysis**

Data were analyzed with GraphPad Prism software (La Jolla, California, US) for normality (D'Agostino-Pearson or the Kolmogorov-Smirnov test for small sAMples). Student's *t*-tests or Mann-Whitney tests were used for normal or non-normal data, respectively and two-tailed ANOVA for grouped data (Bonferroni post-test).

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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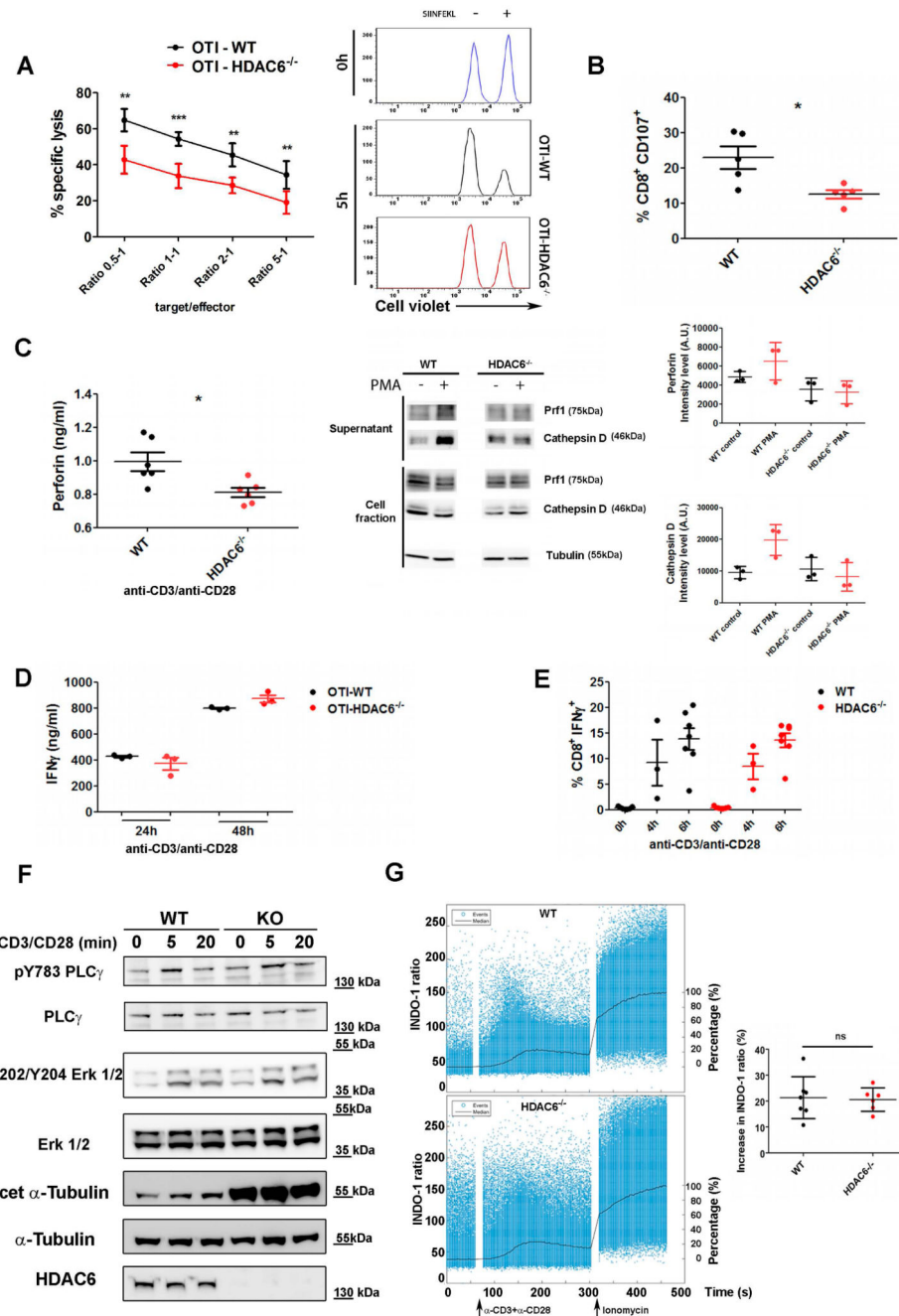
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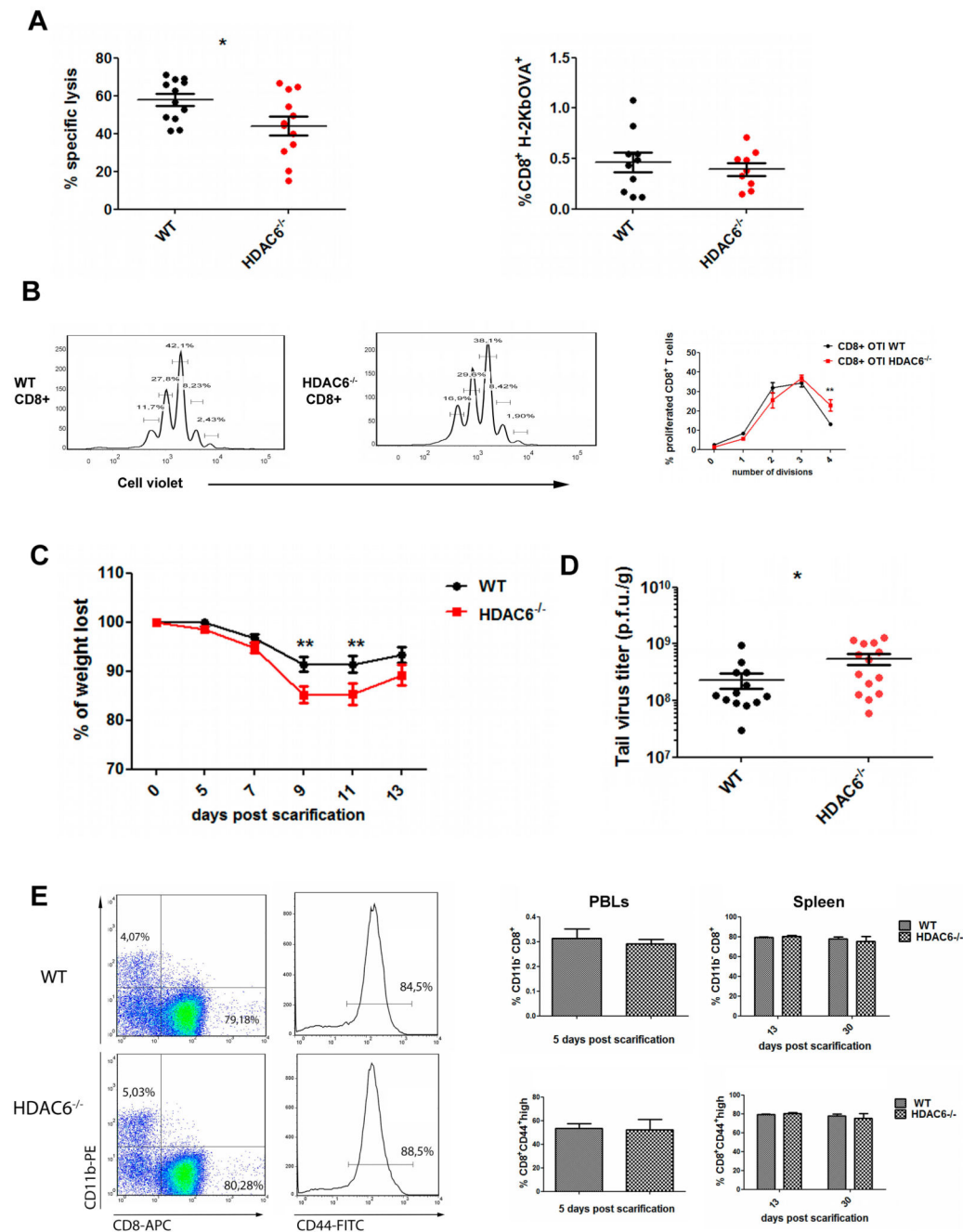


**Figure 1. HDAC6 modulates the efficiency of target cell killing and degranulation of lytic mediators.**

(A) Graph showing *in vitro* cytotoxic assay for specific lysis of SIINFEKL-pulsed EL4 target cells by OT-I-WT or -*Hdac6*<sup>-/-</sup> CTLs. Mean  $\pm$  SEM of specific lysis for 5 h are shown at the indicated target to effector ratios. All killing assay were performed by triplicate. \*, P < 0.05; \*\*, P < 0.01; \*\*\*, P < 0.001. Unpaired T-test (n=5 mice for each genotype).

Histograms, representative FACS profile. (B) Degranulation is shown as the percentage of CD107a<sup>+</sup> cells detected by FACS in activated vs non-stimulated WT and *Hdac6*<sup>-/-</sup> CD8<sup>+</sup> CTLs. Results are mean  $\pm$  s.e.m. n=5 for each genotype. (C) Exocytosis of lytic mediators

upon T cell activation. Left, Perforin content in culture cell supernatants was determined by ELISA. CTLs were stimulated with anti-CD3 and anti-CD28 monoclonal antibodies and 'goat' anti-Armenian-hamster 'IgG' anyibodies (5 h). Graphs show mean $\pm$ s.e.m. (n=6 mice for each genotype). \*P<0.05 (unpaired t-test). Middle panel, representative Western blot showing exocytosis. PMA stimulation (1 h). Normalization was performed against cell fractions. Right graphs, Mean  $\pm$ SEM (3 independent experiments). (D) Graph, IFN $\gamma$  secretion supernatants from anti-CD3/anti-CD28 mAbs activated WT and *Hdac6*<sup>-/-</sup> CTLs by ELISA. Results are mean $\pm$ s.e.m. n=3 for each genotype. Mann-Whitney test. (E) Percentage of IFN $\gamma$ <sup>+</sup> cells in WT and *Hdac6*<sup>-/-</sup> CTLs activated with anti-CD3 and anti-CD28 monoclonal antibodies. Results are mean $\pm$ s.e.m. n=6 (0 and 6 h) and n= 3 (4 h) for each genotype. Mann-Whitney test. (F) Effector CD8<sup>+</sup> CTLs isolated from WT and *Hdac6*<sup>-/-</sup> mice were activated (anti-CD3/anti-CD28 mAbs), lysed and blotted against PLC $\gamma$ 1 (pY783) and erk1/2 (pT202/Y204) phosphorylation and tubulin acetylation. (G) Calcium flux. WT and *Hdac6*<sup>-/-</sup> purified CTLs pre-loaded with the INDO-1 AM probe were analyzed for free and bound Ca<sup>2+</sup> by flow cytometry. Left graphs, time course ratiometric variation (left Y axis). Black line, Median function normalized to basal (0%) and total activation by ionomycin treatment (100%; right Y axis). Stimuli are indicated. A representative experiment is shown. Right graph, Mean of the increase in the ratio  $\pm$ SD (WT, n=7; *Hdac6*<sup>-/-</sup> n=6. Mann Whitney test).



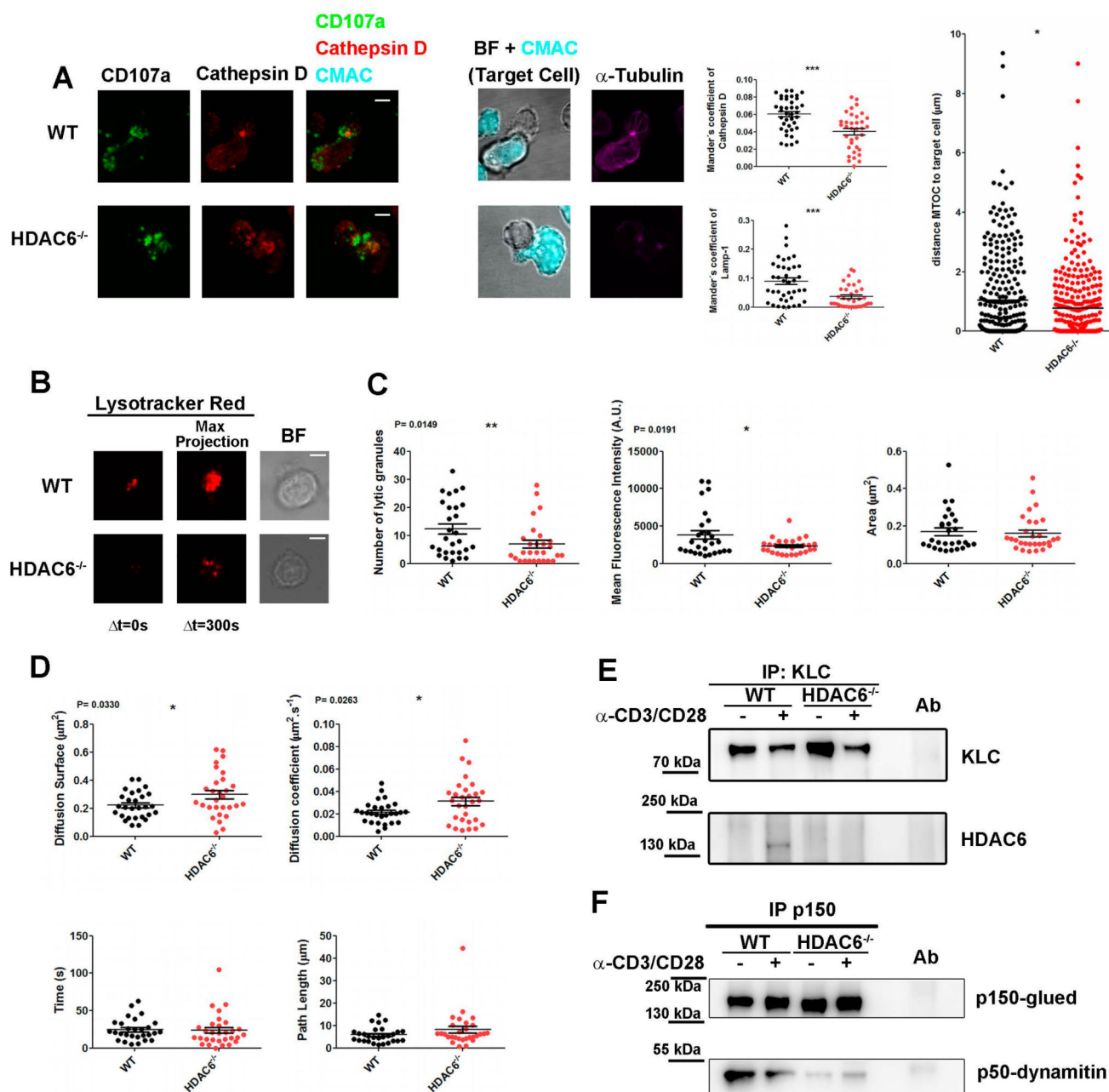
**Figure 2. In vivo function of CTLs is impaired in HDAC6 knockout mice.**

(A) WT and *Hdac6*<sup>-/-</sup> were immunized against SIINFEKL peptide. 7 days after immunization, cell violet-labelled, SIINFEKL-pulsed or not target cells (1  $\mu$ M) were injected i.p., recovered through intraperitoneal lavage (24 h) and cell survival assessed by FACs (left panel). Data represent mean $\pm$ s.e.m. (n= 12 for each genotype from three independent experiments). \*P<0.05, Mann-Whitney test. Relative percentage of CD8<sup>+</sup> H-2Kb<sup>+</sup> was determined to control avidity towards SIINFEKL (right panel, results are mean  $\pm$ s.e.m. (WT, n= 10; *Hdac6*<sup>-/-</sup>, n= 9 from three independent experiments. Mann-Whitney's



test). (B) Proliferation of CD8<sup>+</sup> cells in VACV-OVA infected WT and *Hdac6*<sup>-/-</sup> mice. Results are means±s.e.m. (n= 5 for each genotype). \*\*P<0.01 (Mann-Whitney test). (C) *Rag1*<sup>-/-</sup> mice inoculated i.v. with 0.8x10<sup>6</sup> CD8<sup>+</sup> naïve cells and infected with VACV-WR by tail scarification were weighted every 2 days. Results are means±s.e.m. (n=10 for each genotype). \*\*P<0.01 (Mann-Whitney test). (D) Titration of viral particles from scarified tails (13 d.p.i). The colonies of CV-1 cells infected *in vitro* with different dilutions of tail extracts were counted, and normalized to the tail tissue weight. Results are means±s.e.m. (WT, n= 13; *Hdac6*<sup>-/-</sup> n= 14). \*P<0.05 (Mann-Whitney test). (E) Percentage of CD8<sup>+</sup> expansion and CD44 expression by FACs analysis in peripheral blood (5 d.p.i) and in spleen populations (13 and 30 d.p.i.). CD11b was used as negative control. Results are means ±s.e.m. (n= 5) (Mann-Whitney test).





**Figure 3. HDAC6 drives the terminal transport of LG to the target cell.**

(A) Confocal microscopy images of WT and *Hdac6*<sup>-/-</sup> OT-I-derived CTLs conjugated with CMAC-loaded, OVA pre-pulsed target cell EL4 (500 nM SIINFEKL, Cyan) for 15 min. Green, CD107a. Red, CathepsinD. Magenta,  $\alpha$ -tubulin. Bright-field (BF) images correspond to a unique plane and fluorescence images, to maximal projections from Z-stacks. Middle graphs, Mander's coefficient for co-localization of Lamp1 and CathepsinD. Results are mean  $\pm$  s.e.m. (WT, n=39; *Hdac6*<sup>-/-</sup>, n=35). \*\*\*P<0.001 (Mann-Whitney test). Right graph, quantification of MTOC translocation. Results are mean  $\pm$  s.e.m. (n>270, from 3 independent experiments); \*P<0.05 (unpaired *t*-test). (B) Representative TIRFm images of Lysotracker

Red-loaded WT and *Hdac6*<sup>-/-</sup> CTLs in glass-bottom chambers coated with anti-CD3 and anti-CD28 monoclonal antibodies. Video recording was initiated upon cell adhesion (37°C and 5% CO<sub>2</sub>). Images were acquired for 5 min every 0.5 s. (C-D) Quantification for LG parameters at the IS-like was performed for each cell. Results are means±s.e.m. (n=28 for each genotype, 3 independent experiments). \*P<0.05; \*\*P<0.01. Mann-Whitney test. (E-F) Western blots showing immunoprecipitates from resting or activated WT and *Hdac6*<sup>-/-</sup> CTLs (15 min) from a representative experiment out of three. Antibodies anti-KLC1 (E) or anti-p150 Glued (p150) were used (F). Samples were blotted against indicated antibodies. Ab, pre-immune control antibody. Scale bars: 4 µm.

# The PDZ-adaptor protein syntenin-1 regulates HIV-1 entry

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**ABSTRACT** Syntenin-1 is a cytosolic adaptor protein involved in several cellular processes requiring polarization. Human immunodeficiency virus type 1 (HIV-1) attachment to target CD4<sup>+</sup> T-cells induces polarization of the viral receptor and coreceptor, CD4/CXCR4, and cellular structures toward the virus contact area, and triggers local actin polymerization and phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>) production, which are needed for successful HIV infection. We show that syntenin-1 is recruited to the plasma membrane during HIV-1 attachment and associates with CD4, the main HIV-1 receptor. Syntenin-1 overexpression inhibits HIV-1 production and HIV-mediated cell fusion, while syntenin depletion specifically increases HIV-1 entry. Down-regulation of syntenin-1 expression reduces F-actin polymerization in response to HIV-1. Moreover, HIV-induced PIP<sub>2</sub> accumulation is increased in syntenin-1-depleted cells. Once the virus has entered the target cell, syntenin-1 polarization toward the viral nucleocapsid is lost, suggesting a spatiotemporal regulatory role of syntenin-1 in actin remodeling, PIP<sub>2</sub> production, and the dynamics of HIV-1 entry.

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## INTRODUCTION

The adaptor protein syntenin-1 was originally described in relation to its association with the syndecan receptor and its recycling

(Grootjans *et al.*, 1997; Zimmermann *et al.*, 2005). It was also termed melanoma differentiation-associated gene-9 (mda-9) for its up-regulated expression in several types of tumors, which correlates with high invasiveness (Sarkar *et al.*, 2004, 2008). Structurally, syntenin-1 contains a regulatory N-terminal region and two tandem postsynaptic density protein, *Drosophila* disk large, and zonula occludens-1 (PDZ) domains. The N-terminal region is composed of two Tyr-based domains: one ITIM and one ITAM domain, the ITIM domain being important for CXCR4-mediated T-cell migration (Sala-Valdes *et al.*, 2012). PDZ domains are involved in the formation of macromolecular complexes that often link transmembrane proteins to the actin cytoskeleton, mediating their clustering and polarized subcellular distribution (Fanning and Anderson, 1999; Brone and Eggermont, 2005; Hirbec *et al.*, 2005; Ludford-Menting *et al.*, 2005). PDZ domains interact with short amino acid sequences at the C-terminal end of plasma membrane proteins. Three consensus sequences bind PDZ domains: class I ([S/T]-X-Φ), class II (Φ-X-Φ), and class III ([D/E]-X-Φ), where X is any amino acid and Φ is a hydrophobic residue (Chimura *et al.*, 2011). Through its PDZ domains, syntenin-1 binds to transmembrane proteins, such as CD6 (Gimferrer *et al.*, 2005) and the tetraspanin CD63 (Latysheva *et al.*, 2006); signaling proteins, such as the tyrosine kinase Src (Boukerche *et al.*, 2008);

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Abbreviations used: APC, antigen presenting cell; BSA, bovine serum albumin; CD4, cluster of differentiation-4; CMTMR, 5-(and-6)-((4-chloromethyl)benzoyl)amino tetramethylrhodamine; CNBr, cyanogen bromide; CXCR4, C-X-C chemokine receptor type-4; ELISA, enzyme-linked immunosorbent assay; ENV, viral envelope glycoprotein complex gp120/gp41; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; GFP, green fluorescent protein; GST, glutathione S-transferase; HA, hemagglutinin; HIV-1, human immunodeficiency virus type 1; ITAM, immunoreceptor tyrosine-based activating motif; ITIM, immunoreceptor tyrosine-based inhibitory motif; mda-9, melanoma differentiation-associated gene-9; PDZ, postsynaptic density protein, *Drosophila* disk large, and zonula occludens-1; PI4P5K-1α, phosphatidylinositol-4-phosphate-5 kinase type I-α; PIP<sub>2</sub>, phosphatidylinositol 4,5-bisphosphate; PLC-δ-PH-GFP, pleckstrin homology domain of phospholipase C-δ tagged to GFP protein; siRNA, small interfering RNA; TBS, Tris-buffered saline; VLP, virus-like particle; VSV, vesicular stomatitis virus.

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and the actin-linker protein merlin (Jannatipour *et al.*, 2001). Recruitment of syntenin-1 to the plasma membrane is also regulated through its PDZ-mediated interaction with the lipid second messenger phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>; Zimmermann, 2006).

Syntenin-1 has a cytosolic distribution, being enriched in vesicles and F-actin structures (Zimmermann *et al.*, 2001). It is implicated in several actin-polarized processes, such as cell migration, immune synapse formation, intracellular trafficking, cell-surface targeting, axonal outgrowth, and synaptic transmission (Gimferrer *et al.*, 2005; Hirbec *et al.*, 2005; Zimmermann *et al.*, 2005; Beekman and Coffey, 2008; Sarkar *et al.*, 2008). We recently reported the involvement of syntenin-1 in CXCR4-mediated T-chemotaxis and T-APC antigen presentation, in which it regulates Rac-triggered actin polymerization (Sala-Valdes *et al.*, 2012).

CD4/CXCR4-derived signaling and Rac-induced actin remodeling are also important for human immunodeficiency virus type 1 (HIV-1) infection (Pontow *et al.*, 2004; Carter *et al.*, 2009). Soon after HIV-1 contact with CD4<sup>+</sup> T-cells, the viral envelope glycoprotein complex gp120/gp41 (Env) engages with CD4 and CXCR4, inducing several signaling pathways that trigger receptor clustering (Jimenez-Baranda *et al.*, 2007; Barrero-Villar *et al.*, 2009) in a structure called “cap” (Dianzani *et al.*, 1995; Nguyen *et al.*, 2005), local actin polymerization (Iyengar *et al.*, 1998; Jolly *et al.*, 2004), T-cell activation (Popik *et al.*, 1998; Hiscott *et al.*, 2001), and HIV-1 replication (Popik and Pitha, 2000; Mettling *et al.*, 2008). These rearrangements enhance viral entry (Wu and Yoder, 2009; Juno and Fowke, 2010) by supplying an optimal density of CD4/CXCR4 complexes through actin-mediated surface reorganization (Iyengar *et al.*, 1998; Doms, 2000; Jolly *et al.*, 2004). Thus the actin-binding proteins moesin and filamin-A accumulate at the F-actin-enriched capping areas and influence CD4/CXCR4 clustering (Jimenez-Baranda *et al.*, 2007; Barrero-Villar *et al.*, 2009). Moreover, we previously showed that HIV-1 gp120, through its interaction with CD4, induces local production of PIP<sub>2</sub> by activating PI4P5K-1 $\alpha$  (Barrero-Villar *et al.*, 2008).

After viral attachment to the plasma membrane, the local F-actin accumulation can constitute a physical barrier for entry of the viral nucleocapsid (Vasiliver-Shamis *et al.*, 2009). In this regard, CXCR4-gp120 interaction was recently shown to activate cofilin, which promotes actin clearance, facilitating nucleocapsid entry into the cytoplasm (Yoder *et al.*, 2008).

In this study, we assessed the possible role of syntenin-1 as a mediator of CD4/CXCR4 clustering and actin remodeling during HIV-1 infection. Our data indicate that syntenin-1 associates with CD4 and negatively regulates HIV-1 infection by affecting the dynamic reorganization of actin cytoskeleton and PIP<sub>2</sub> production triggered upon HIV-1 contact, specifically at the viral entry step.

## RESULTS

### Syntenin-1 is recruited to the plasma membrane by HIV-1 Env

PDZ proteins commonly determine the polarization of cellular events (Fanning and Anderson, 1999; Brone and Eggermont, 2005; Ludford-Menting *et al.*, 2005). Because HIV-1 contact with target cells triggers the polarization of cellular components (Dianzani *et al.*, 1995; Nguyen *et al.*, 2005), we investigated whether the PDZ protein syntenin-1 was recruited upon HIV-1 contact with target CD4<sup>+</sup> T-cells (CEM-T4). In the absence of HIV-1 virus, syntenin-1 displayed a diffuse cytosolic pattern with low clustering at the plasma membrane (Figure 1A). In contrast, after incubation of T-cells with free HIV-1 particles, syntenin-1 was clearly recruited toward the CD4/CXCR4 capping area (Figure 1, A and E). To confirm the specificity

of syntenin-1 recruitment, we assessed CD45 distribution and observed that it did not accumulate at virus-induced capping areas (Figure 1, B and E). In addition, to assess that syntenin-1 recruitment was Env-mediated, target CEM-T4 cells were incubated with a Jurkat clone expressing the T-tropic HIV-1 envelope HxB (Env<sup>+</sup> HxBc2 cells). Syntenin-1 was found at the Env-driven cell contacts, together with CD4 (Figure 1, C and E). We also cocultured target CEM-T4 cells with T-cells directly infected with HIV–green fluorescent protein (GFP) virus (MT4-HIV-GFP). In these cocultures, syntenin-1 accumulated at cell–cell contacts, areas in which viral synapses are established (Figure 1, D and E), but not at unspecific Env-independent cell–cell contacts (Figure 1E, right-hand white bar; exemplified in Supplemental Figure S1). Quantification confirmed significant accumulation of syntenin-1 at contacts with HIV-1 viral particles and infected cells, in comparison with CD45 (Figure 1E).

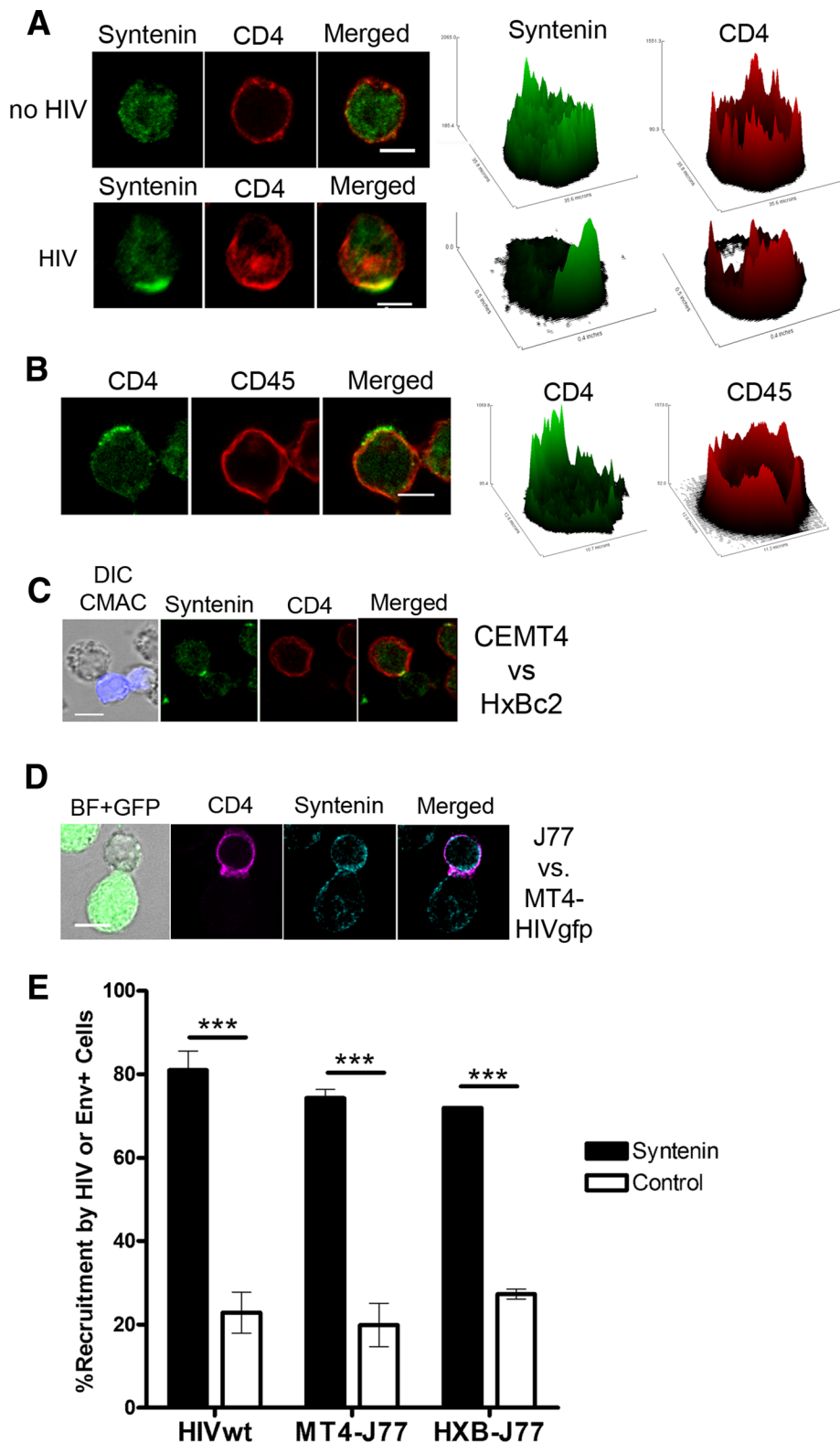
### Syntenin-1 associates with the cytoplasmic tail of CD4

PDZ domains usually bind to specific consensus sequences at the C-terminal ends of transmembrane proteins (Chimura *et al.*, 2011). To determine whether syntenin-1 forms part of the CD4/CXCR4 complex at the capping areas, we first searched the CD4 and CXCR4 cytoplasmic domain sequences for potential PDZ-binding motifs. A class I PDZ-binding consensus sequence (Ser-Pro-Ile) was detected at the C-terminal end of CD4, suggesting potential direct interaction with syntenin-1 via its PDZ domains. To test this, we immobilized glutathione S-transferase (GST)-fused hemagglutinin (HA)- or Myc-tagged syntenin-1 on Sepharose beads, and incubated them with human primary lymphoblast lysates. GST-syntenin-HA and GST-syntenin-Myc, but not GST alone, precipitated CD4 (Figure 2A). Confirming this interaction, anti-CD4 antibody immunoprecipitated endogenous syntenin-1 from CEM T-cell lysates; syntenin-1 was detected with anti-syntenin-1 antibody as a double band of around 32 kDa (Figure 2B). To demonstrate the occurrence of CD4–syntenin-1 association in the context of HIV-1 infection, we performed reverse immunoprecipitation assays with Jurkat T-cells previously incubated with free viral particles. Immunoprecipitation of syntenin-1, followed by immunodetection of CD4, detected constitutive CD4–syntenin-1 interaction that was maintained during HIV-1 contact (Figure 2C). Together, these data suggest that syntenin-1 interacts with CD4 before and during HIV-1 infection.

### Syntenin-1 overexpression inhibits HIV-1-induced cell fusion and viral production

To assess the specific role of syntenin-1 during HIV-1 infection, target T-cells were transfected with GFP-tagged, wild-type syntenin-1 (syntenin-GFP) or a mutated protein, syntenin-Y1-GFP (Synt-Y1-GFP), which carries a Y4>F point mutation in the ITIM motif of the regulatory N-terminal region. Phosphorylation of endogenous syntenin-1 at this tyrosine residue has been shown to be essential for CXCR4-induced chemotaxis (Sala-Valdes *et al.*, 2012). Syntenin-GFP and Synt-Y1-GFP showed a similar distribution to endogenous syntenin-1, being enriched at Env-driven cell–cell contacts (Figure 3A) and HIV-induced capping areas (Figure 3B and data not shown). Target J77 cells overexpressing these GFP-tagged proteins were infected with free HIV-1 particles, and viral production was measured 3 d later by p24 enzyme-linked immunosorbent assay (ELISA). Cells overexpressing syntenin-GFP produced lower viral titers than control cells (transfected with GFP alone). In contrast, overexpression of the mutated syntenin-1 had no effect on HIV-1 production (Figure 3C).

To investigate the potential effect of syntenin-1 plasma membrane recruitment on HIV-1-induced cell fusion, we measured Env-mediated cell fusion by flow cytometry. Env<sup>+</sup> HxBc2 cells



**FIGURE 1:** Syntenin-1 is recruited by HIV-1 envelope glycoproteins. (A) CEM-T4 cells incubated with or without HIV-1 particles for 30 min were fixed and immunostained for syntenin-1 and CD4 (HP2/6). Summative projections of confocal stack images are shown. Scale bars: 5  $\mu$ m. Surface plots show fluorescence distribution and intensity for corresponding images. (B) CEM-T4 cells incubated with HIV-1 particles for 30 min were fixed and immunostained for CD4 (CD4v4-FITC) and CD45 (D3/9). Summative projections of confocal stack images are shown. Scale bar: 5  $\mu$ m. Surface plots show fluorescence distribution and intensity for corresponding images. (C) Immunofluorescence microscopy images of target CEMT4 cells incubated for 2 h with Env<sup>+</sup> HxBc2 cells (CMAC stained, blue) and stained for syntenin-1 and CD4 (HP2/6). Scale bars: 5  $\mu$ m.

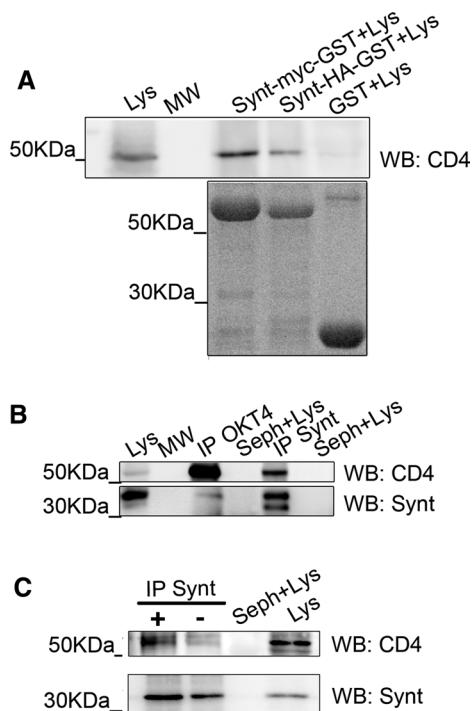
were labeled with red intracellular tracker (CMTMR; 5-(and-6)-(((4-chloromethyl)benzoyl)-amino) tetramethylrhodamine) and cocultured with target T-cells transfected with each GFP-tagged syntenin-1 construct. Env-driven cell fusion leads to syncytia formation, detected as double-fluorescence positive events of higher size and complexity. As a control of specificity, syncytia formation was blocked with the HIV-1 fusion inhibitor T-20 (Figure 3D). Quantification of these events showed that overexpression of wild-type syntenin-1 reduced Env-mediated cell fusion, whereas the Y4>F mutant had no significant effect (Figure 3D). These data suggest that syntenin-1 negatively regulates HIV-1-mediated membrane fusion through a mechanism requiring phosphorylation of tyrosine 4 in the ITIM motif. To assess whether the Y4>F point mutant was able to interact with CD4, cells were transfected with syntenin-GFP or Synt-Y1-GFP, immunoprecipitated with an anti-GFP, and blotted for CD4. As shown in Figure 3E, Synt-Y1-GFP also associated with CD4.

#### Knockdown of syntenin-1 expression increases HIV-1 cell fusion and viral entry

To further study syntenin-1 function during HIV-1 infection, we knocked down endogenous syntenin-1 expression using small interfering RNA (siRNA). Control cells were transfected with an siRNA sequence that does not hybridize with any eukaryotic mRNA. Syntenin-1 down-regulation was assessed for each experiment (Figure 4A and data not shown). Membrane expression levels of CD4 and CXCR4 were also routinely assessed to confirm that they were not affected by syntenin depletion (Figure 4B). To assess Env-driven cell fusion, silenced T-cells loaded with green cell tracker were cocultured with

(D) Target J77 cells were incubated for 2 h with HIV-1-infected T-cells (MT4-HIV-GFP) and immunostained for syntenin-1 and CD4 (HP2/6). Projections of confocal stack images are shown. Scale bar: 5  $\mu$ m. (E) Quantification of syntenin-1 accumulation at HIV-1-induced capping areas (HIV-wt), contacts between target cells and HIV-infected cells (MT4-J77), or contacts between target cells and Env<sup>+</sup> cells (HxB-J77). The control (white bars) for HIV wild-type and MT4-J77 represent the recruitment of CD45. The control (white bar) for HxB-J77 corresponds to the recruitment of syntenin at Env-independent cell-cell contacts (between two target J77 cells). The chart represents the mean  $\pm$  SD of three independent experiments; more than 100 conjugates were counted in each condition.





**FIGURE 2:** Syntenin-1 interacts with CD4 during HIV infection of T-cells. (A) T-lymphoblast cell lysates (Lys) were pulled down with GST, GST-syntenin-Myc, or GST-syntenin-HA, and blotted with anti-CD4 mAb MEM241. (B) CEM T-cell lysates were immunoprecipitated with anti-CD4 mAb OKT4 (IP-OKT4) and anti-syntenin pAb (IP-Synt), and then blotted for syntenin-1 (pAb) and CD4 (mAb MEM241). Total lysates (Lys) and Sepharose beads incubated with lysates (Seph+Lys) are shown. (C) Jurkat J77 T-cells incubated with or without HIV-1 particles were lysed, immunoprecipitated with anti-syntenin-1 pAb, and blotted with anti-CD4 mAb MEM241.

Env<sup>+</sup> HxBc2 cells labeled with red cell tracker. Syntenin-1-depleted cells exhibited enhanced cell fusion with Env<sup>+</sup> cells compared with control cells (Figure 4C). Furthermore, syntenin-1-depleted cells produced higher viral titers than control cells upon infection with free HIV-1 virions (Figure 4D). Importantly, this effect could be rescued by syntenin-GFP overexpression in silenced T-cells (Figure 4E). Taken together, these results confirm the inhibitory role of syntenin-1 in HIV-1 infection.

### Syntenin-1 restricts HIV-1 entry

Syntenin-1 recruitment at the plasma membrane suggested its involvement during HIV-1 attachment or entry steps. To distinguish between these possibilities, we incubated syntenin-1-depleted cells with HIV-1 particles for 2 h at 4°C, a temperature that does not allow Env-induced membrane fusion, thus arresting the virus at the attachment step, or at 37°C, a permissive temperature for membrane fusion and consequently for viral entry. For both conditions a low infection rate was used to avoid superinfection and cooperative virion entry. The amount of p24 in the lysates was measured by ELISA (Figure 5A) and Western blot (data not shown); HIV-1 attachment was estimated from the p24 level at 4°C, and HIV entry was estimated from the difference between the levels at 37 and 4°C. Syntenin-1 silencing enhanced virus entry without affecting viral attachment (4°C), suggesting that syntenin-1 specifically impairs HIV entry (Figure 5A). To confirm these observations, we infected target cells with a one-cycle luciferase virus displaying a T-tropic envelope (X4-Luc), or the vesicular stomatitis virus (VSV) envelope (VSV-Luc) as a control.

Higher luciferase levels were found in X4-Luc-infected syntenin-1-silenced cells, confirming that syntenin-1 knockdown specifically increases viral entry (Figure 5B). VSV infection was similar in silenced and control cells, demonstrating that the effect observed is HIV-1 Env-dependent (Figure S2). These results indicate that syntenin-1 negatively regulates HIV-1 entry without affecting viral attachment.

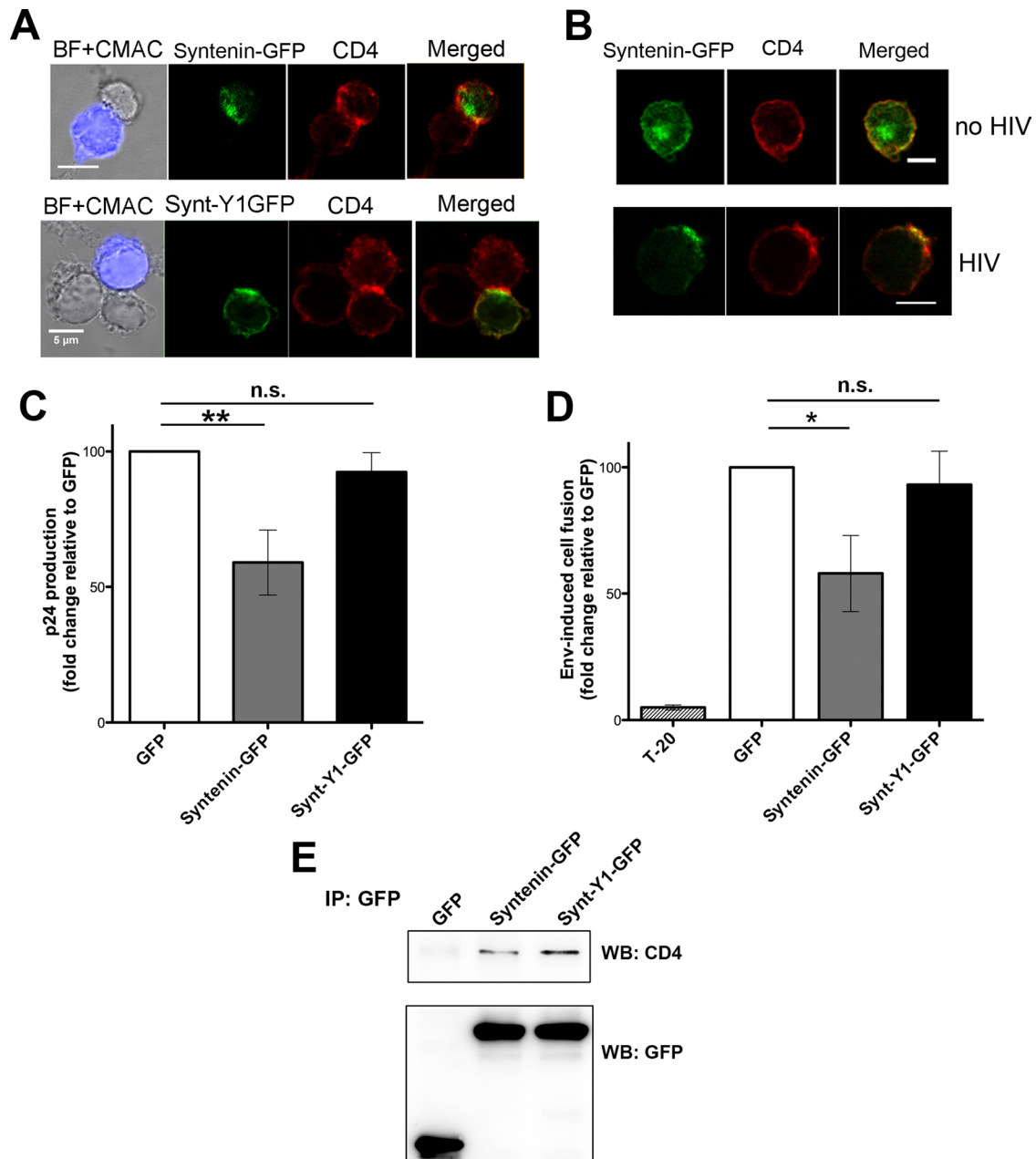
To study the behavior of syntenin-1 during viral attachment and entry in more detail, we incubated Gag-GFP fluorescent virus-like particles (VLPs) with T-cells at 4°C or 37°C. At 4°C, VLPs attached preferentially to one pole of the target cell (Figure S3; see Supplemental Movie S1 for three-dimensional reconstruction), which suggests that the capping area behaves as the main site for viral entry, as has been proposed (Doms, 2000; Nguyen et al., 2005). At 37°C, VLPs could be observed inside target cells, usually accumulated at one pole of the cytoplasm (Figure S3; see Movie S2 for three-dimensional reconstruction).

We next examined the localization of endogenous syntenin-1 by confocal immunostaining of T-cells exposed to VLPs. To avoid false positives due to fortuitous superposition, we only analyzed planes in which VLPs were detected (usually one to two planes distanced by 0.25 μm). Syntenin-1 was enriched at VLP attachment sites in cells kept at 4°C (Figure 5, C and F), whereas barely any syntenin-1 localized at the internalized VLPs in cells incubated at 37°C (Figure 5, D and F). However, syntenin-1 did accumulate near VLPs positioned at cell-cell contacts at 37°C (Figure 5, E and F). Quantification of syntenin-1 accumulation at VLPs at each temperature and location confirmed that association of syntenin-1 with VLPs occurs only on the plasma membrane, and not once the particles are already internalized (Figure 5F).

Thus syntenin-1 is recruited to the plasma membrane by HIV-1 Env during the attachment and entry steps but affects only the effectiveness of viral entry, and it is released from the viral complex once the nucleocapsid has entered the cytoplasm.

### Syntenin-1 regulates actin polymerization and PIP<sub>2</sub> accumulation upon HIV-1 contact

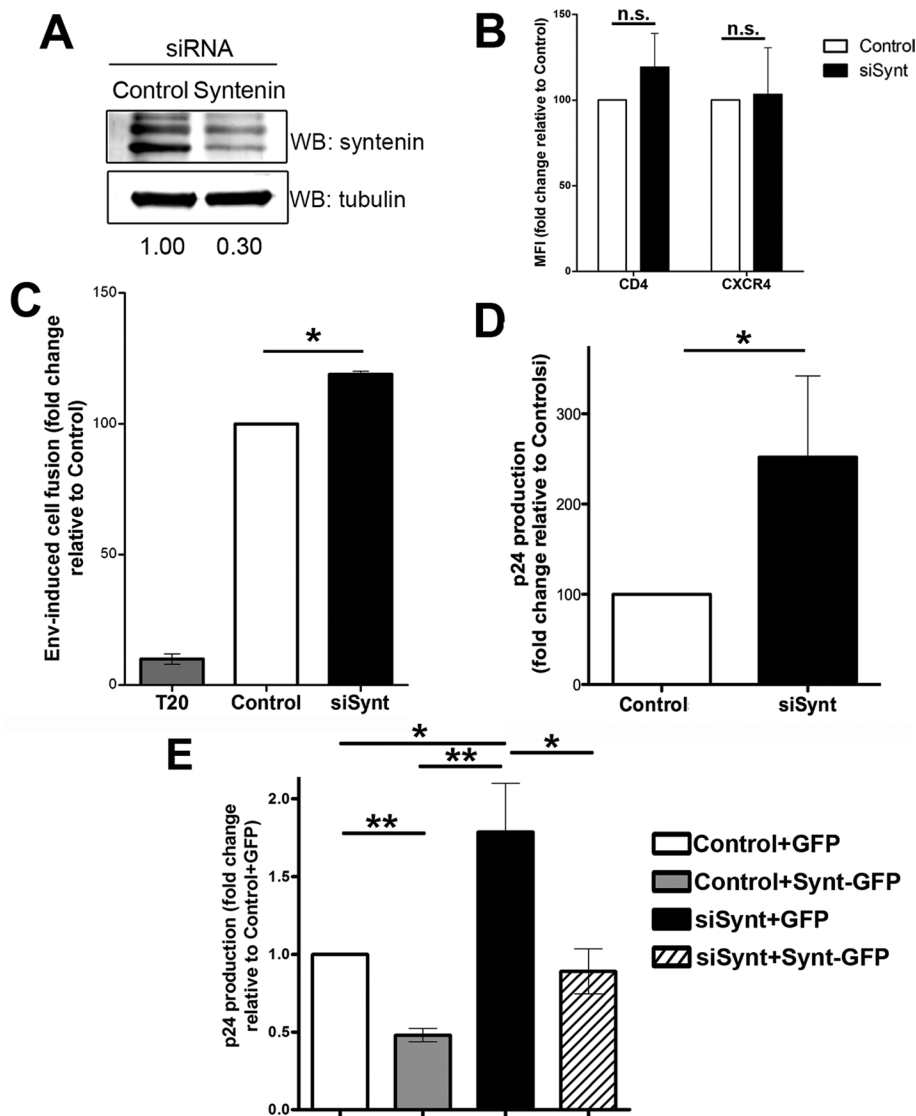
Syntenin-1 was recently found to regulate F-actin polymerization via Rac GTPase in T-cells during CXCR4-induced chemotaxis and superantigen presentation (Sala-Valdes et al., 2012). This syntenin-1-mediated activation of Rac depends on phosphorylation of tyrosine 4 (Sala-Valdes et al., 2012). Moreover, Rac-mediated actin reorganization is known to be essential for HIV-1 entry (Iyengar et al., 1998; Pontow et al., 2004; Harmon et al., 2010). To analyze the relation between syntenin-1 and F-actin during HIV-1 infection, we incubated target cells with VLPs, stained them for syntenin-1 and F-actin, and analyzed them with confocal microscopy. Syntenin-1 clusters at the plasma membrane corresponded with the VLP contact area and partially overlapped with F-actin accumulation (Figure 6A; see Figure S4 for F-actin staining in noninfected target cells). Given that HIV-1 attachment to CD4 and CXCR4 triggers local actin polymerization (Jolly et al., 2004), we analyzed this process in syntenin-1-depleted cells. A similar percentage of VLPs colocalized with F-actin in control and syntenin-1-depleted cells (Figure 6A). However, when actin polymerization after HIV-1 contact was monitored at different time points, control cells showed a marked increase in F-actin content 30 min after HIV-1 contact, whereas F-actin polymerization was markedly lower in syntenin-1-depleted cells (Figure 6B). These data indicate that syntenin-1 is required for HIV-1-triggered F-actin polymerization at early stages, when most virus particles are observed at the plasma membrane. Moreover, F-actin accumulation at cell-cell contacts between target and infected MT4 cells was reduced in cells knocked down for syntenin in comparison with control cells (Figure 6C).



**FIGURE 3:** Overexpression of wild-type syntenin-1, but not a phosphorylation-defective mutant, in target T-cells impairs HIV-1 infection. (A) CEM-T4 cells overexpressing syntenin-GFP or Synt-Y1-GFP (containing the Y4>F point mutation) were incubated with Env+ HxBc2 cells (tracked with CMAC, blue) for 2 h, fixed, and stained for CD4 (HP2/6 mAb). Summative projections of confocal stack images are shown. Scale bars: 5  $\mu$ m. (B) CEM-T4 cells overexpressing wild-type syntenin-1 (syntenin-GFP) were incubated for 30 min with or without HIV-1 particles, fixed, and stained for CD4 (HP2/6 mAb). Projections of confocal stack images are shown. Scale bars: 5  $\mu$ m. (C) HIV-1 infection is inhibited by syntenin-GFP but not by Synt-Y1-GFP. Infection was measured as viral production, quantified as p24 viral protein content in supernatants (ELISA). Data are the fold induction relative to control cells transfected with GFP alone (mean  $\pm$  SD of four experiments performed in triplicate;  $p = 0.0058$ ). (D) Cell fusion triggered by HIV-1 envelope is reduced in cells overexpressing syntenin-GFP. As a control, syncytia formation was blocked by incubation with the fusion inhibitor T20 (10 nM). Data are the fold induction relative to GFP-transfected cells (mean  $\pm$  SD of four experiments performed in duplicate;  $p = 0.031$ ). (E) Cells were transfected with GFP, syntenin-GFP, or Synt-Y1-GFP; immunoprecipitated with pAb anti-GFP; and blotted for CD4 (MEM241) and GFP (mAb).

Interaction of the HIV-1 envelope with CD4 triggers PIP<sub>2</sub> production (Barrero-Villar *et al.*, 2008), and this lipid has been found to regulate not only syntenin-1 recruitment to the plasma membrane (Zimmermann *et al.*, 2005) but also many other actin-related proteins (Saarikangas *et al.*, 2010). We therefore assessed whether syntenin-1-dependent actin polymerization upon HIV-1 contact was related to

local production of PIP<sub>2</sub>. Control or syntenin-1-depleted cells were incubated with MT4-HIV-GFP cells for 2 h and then fixed and stained for PIP<sub>2</sub> with anti-PIP<sub>2</sub> mAb. Syntenin-1-depleted T-cells showed increased accumulation of PIP<sub>2</sub> at contacts with HIV-1-infected T-cells (Figure 6D). To confirm that this effect was related to HIV-1 Env-dependent contacts, we quantified PIP<sub>2</sub> accumulation in target cells



**FIGURE 4:** Knockdown of syntenin-1 expression increases HIV-1 entry and syncytia formation. (A) Representative Western blot showing silencing of endogenous syntenin-1 expression using oligonucleotide J-008270-08 in T-cells 48 h after siRNA transfection. Numbers below the blot show band intensity ratios between cells transfected with control siRNA and syntenin-1 siRNA, expressed relative to tubulin. (B) Bar chart showing surface expression levels (mean fluorescence intensity) of CD4 and CXCR4 in control and syntenin-depleted cells measured by flow cytometry. (C) Syntenin-1 silencing in target T-cells favors syncytia formation induced by Env<sup>+</sup> HxBc2 cells. Syncytia formation was quantified by flow cytometry detection of double-positive fluorescent cells and is presented as the fold induction relative to cells transfected with control siRNA (mean  $\pm$  SD of four independent experiments performed in duplicate;  $p = 0.017$ ). As a control, syncytia formation was blocked by incubation with the fusion inhibitor T20 (10 nM). (D) Effect of syntenin-1 silencing on HIV-1 infection. Infection was measured as viral production (p24 viral protein content in supernatants [ELISA]) and depicted as the mean fold induction relative to cells transfected with control siRNA (mean  $\pm$  SD of four experiments performed in triplicate;  $p = 0.025$ ). (E) Syntenin-1-GFP overexpression on cells knocked down for the endogenous syntenin-1 rescues the level of HIV-1 infection. Infection was measured as p24 viral protein content in supernatants (ELISA) and quantified as the mean fold induction relative to cells transfected with control siRNA + GFP (mean  $\pm$  SD of two experiments performed in triplicate).

cocultured with Env<sup>+</sup> HxBc2 cells (heterotypic, Env-driven contacts) or cultured alone (homotypic, Env-independent contacts between target cells; Figure 6E and see Figure S5 for an example image). Syntenin-1 knockdown specifically increased PIP<sub>2</sub> accumulation at Env-driven contacts, while having no effect on Env-independent contacts (Figure 6E).

tin polymerization soon after HIV-1 contact, the most plausible explanation for its inhibitory effect is that it induces an actin accumulation at the viral attachment site that hinders viral nucleocapsid entry at the cell surface. Localized actin clearance during HIV-1 entry has been proposed as a mechanism for overcoming this physical restriction (Yoder *et al.*, 2008; Liu *et al.*, 2009; Vasiliver-Shamis *et al.*, 2009), and

To assess whether syntenin-1 depletion increases PIP<sub>2</sub> accumulation in virus–cell contacts, we transfected control and syntenin-1–depleted cells with PLC $\delta$ -PH-GFP (pleckstrin homology domain of PLC- $\delta$  tagged to GFP), a biosensor that binds to PIP<sub>2</sub>-enriched membranes (Varnai and Balla, 1998), and incubated them with Cherry-fluorescent VLPs expressing HxB (T-tropic) envelope. Patches of PLC $\delta$ -PH-GFP were frequently observed on the target cell surface at sites of VLP attachment (a representative image is shown in Figure 6F). The frequency of these events was higher in syntenin-1–depleted cells (Figure 6F). Taken together, these data indicate that syntenin-1 depletion increases PIP<sub>2</sub> accumulation at Env-driven contacts both of cell–cell and of virus–cell interactions, correlating with lower F-actin polymerization and higher viral entry.

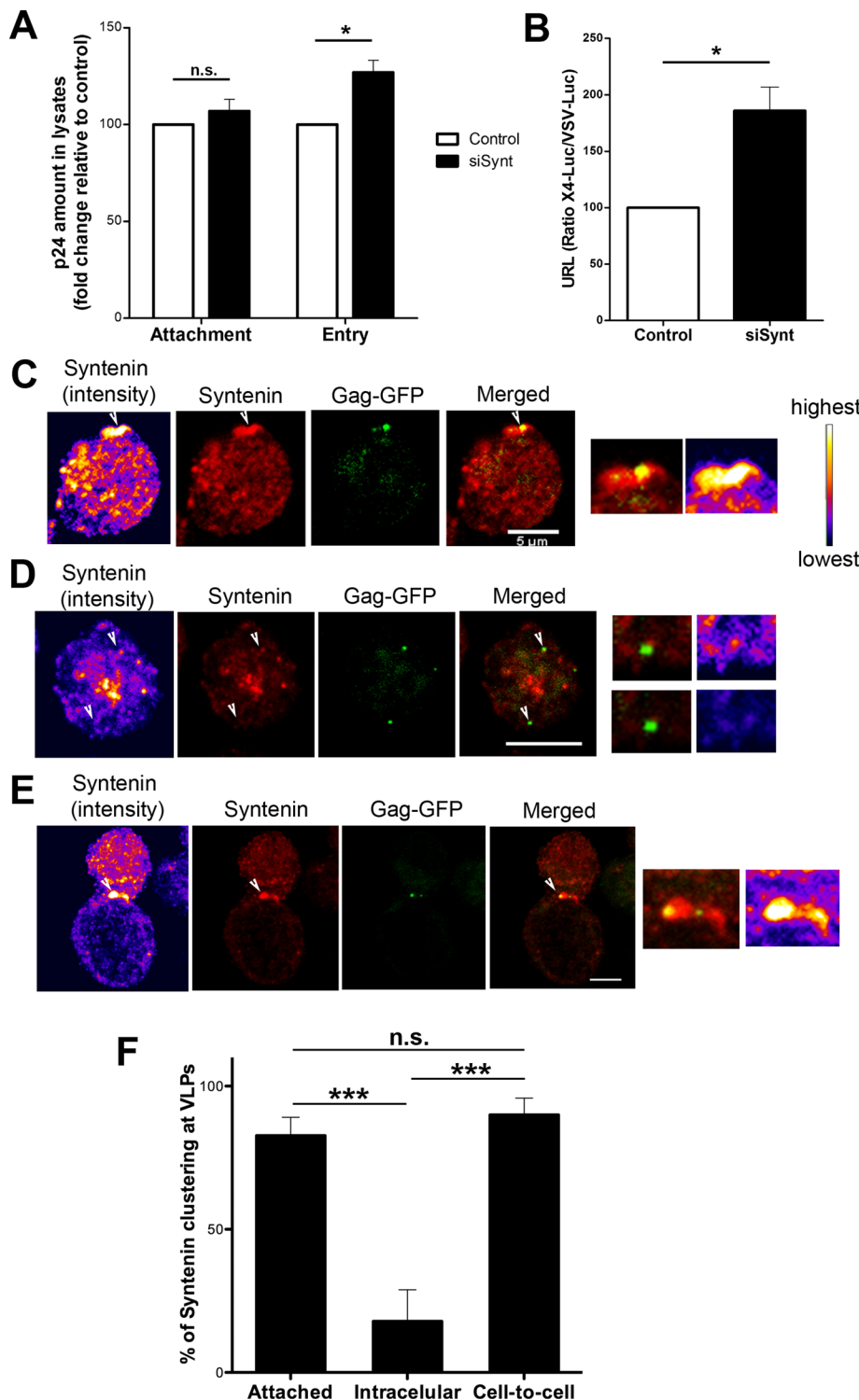
## DISCUSSION

In this study we show that syntenin-1 is recruited toward HIV-1 Env-driven virus–cell and cell–cell contacts, associates with CD4, limits HIV-1–induced cell fusion and viral entry, and modulates Env-triggered F-actin remodeling and PIP<sub>2</sub> production.

HIV-1 binding to CD4/CXCR4 complexes at the T-cell surface activates several signaling pathways and triggers local actin polymerization (Iyengar *et al.*, 1998; Jolly *et al.*, 2004), which is essential for receptor clustering at the plasma membrane (Iyengar *et al.*, 1998; Jolly *et al.*, 2004, 2007; Pontow *et al.*, 2004). The actin-binding proteins filamin-A and ERMs are known to favor CD4/CXCR4 clustering during HIV-1 attachment (Jimenez-Baranda *et al.*, 2007; Barrero-Villar *et al.*, 2009). Filamin-A is needed for a transient cofilin inactivation through a RhoA-ROCK–dependent mechanism that stabilizes F-actin during HIV-1 attachment (Jimenez-Baranda *et al.*, 2007). ERMs are activated by phosphorylation upon HIV-1 contact and are essential for F-actin polarization and CD4/CXCR4 clustering (Barrero-Villar *et al.*, 2009). In contrast with these actin-binding proteins, syntenin-1 does not affect HIV-1 attachment and therefore does not seem to be involved in HIV-1–induced CD4/CXCR4 clustering.

Instead, syntenin-1 binding to CD4 might block Lck activation or other CD4-derived pathways, thus hampering HIV-1 entry. However, since syntenin-1 depletion reduces ac-



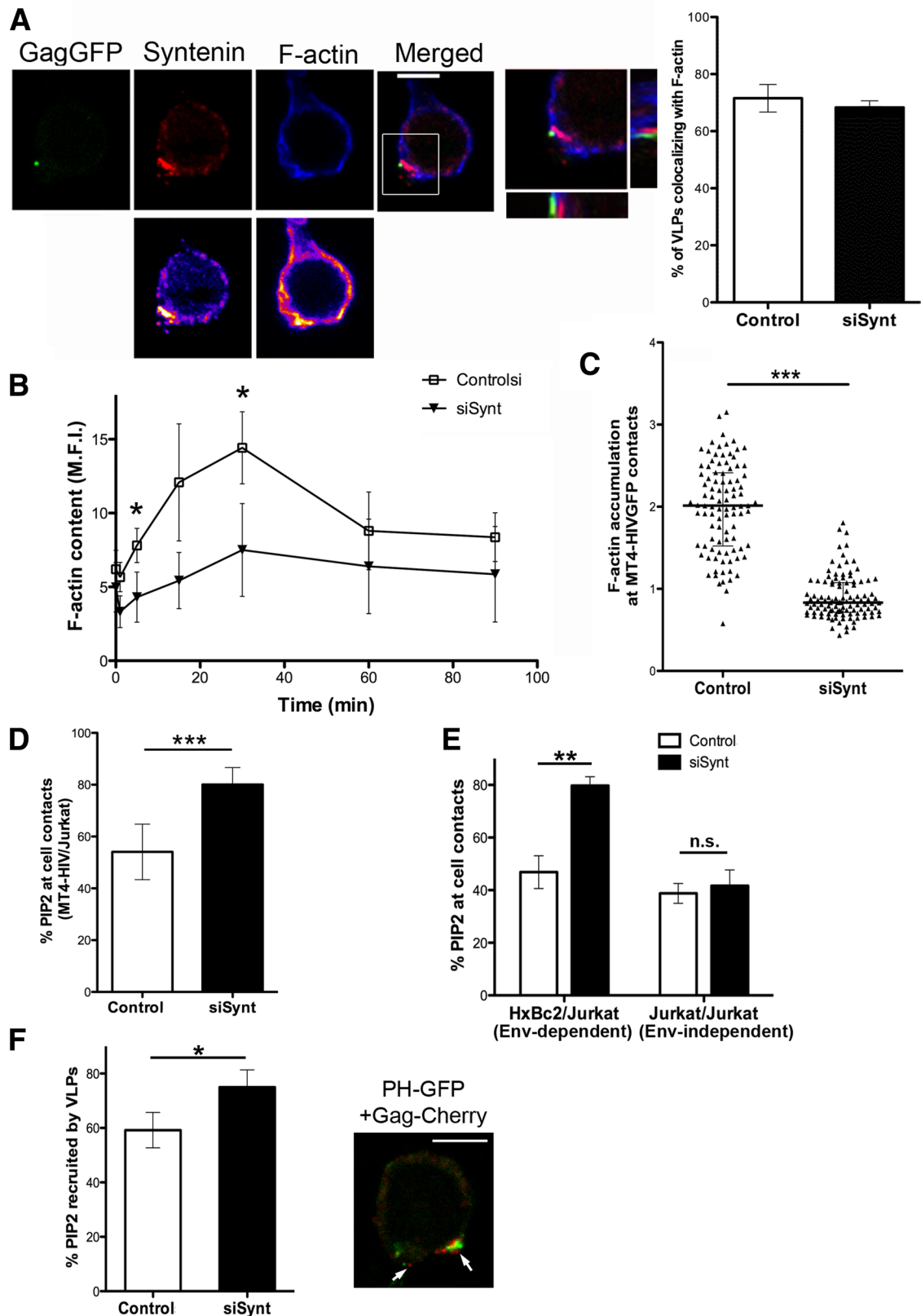


**FIGURE 5:** Syntenin-1 specifically inhibits viral entry and coclusters with fluorescent VLPs at the plasma membrane. (A) Effect of syntenin-1 silencing on HIV-1 attachment (4°C) and entry (37–4°C) into target T-cells. Data present the fold induction relative to control cells (mean  $\pm$  SD of three experiments performed in triplicate;  $p = 0.016$ ). (B) HIV-1 entry in syntenin-silenced cells measured by infection with one-cycle viral particles carrying a luciferase reporter gene. Data present the fold induction of luciferase-HIV-1-envelope virus relative to control cells and normalized to values obtained with luciferase-VSV-envelope virus (mean  $\pm$  SD from four independent experiments performed in triplicate;  $p = 0.038$ ). (C) Confocal images showing the recruitment of endogenous syntenin-1 toward attached VLPs (Gag-GFP) at 4°C. Images are projections of the planes in which each individual VLP is observed. Scale bar: 5  $\mu$ m. Syntenin-1 staining is shown in pseudocolor intensity-coding format next to the confocal image. The small panels are zoomed views of VLP contacts from pseudocolor syntenin-1 and merged

cofilin activation and its actin-severing function have been linked to enhanced HIV entry (Yoder *et al.*, 2008). Thus a reduced F-actin structure after HIV-1 attachment renders cells more susceptible to subsequent HIV-1 nucleocapsid entry, as occurs in syntenin-depleted cells.

Higher HIV-1-mediated cell fusion and viral entry in syntenin-1-depleted target T-cells also correlates with increased PIP<sub>2</sub> production at Env-mediated cell-cell contacts. PIP<sub>2</sub> is an important second messenger that targets proteins to the plasma membrane and regulates many actin-related proteins, such as ERMs, filamin, cofilin, and  $\alpha$ -actinin (Saarikangas *et al.*, 2010). Generation of PIP<sub>2</sub> triggers local actin cytoskeleton changes by activating ERMs (Nakamura *et al.*, 1999), recruiting cofilin (van Rheenen *et al.*, 2009), and inhibiting  $\alpha$ -actinin bundling activity (Corgan *et al.*, 2004). Overall, these pathways might favor HIV-1 membrane fusion by facilitating receptor clustering and subsequent actin clearance (Liu *et al.*, 2009). Remarkably, although PIP<sub>2</sub>-associated cofilin is inactive in tumor cells, cofilin activation in leukocytes is mainly regulated by dephosphorylation (van Rheenen *et al.*, 2009), which is in fact induced upon HIV-1 contact through CXCR4-derived signaling (Yoder *et al.*, 2008). Thus PIP<sub>2</sub> might switch the association of membrane receptors and their cytosolic partners (Zimmermann *et al.*, 2005; Saarikangas *et al.*, 2010), modulating the links between transmembrane proteins and the subcortical actin cytoskeleton, and subsequently altering the association of proteins belonging to the same signaling pathway. In this

immunostaining images ( $\times 3$ ). Note that the maximum intensity of syntenin-1 staining (white on the pseudocolor scale images) overlaps the VLP attachment site. (D) Confocal images showing endogenous syntenin-1 distribution in target T-cells with internalized VLPs (37°C). Two VLPs are shown: one with low syntenin-1 clustering and another with no clustering. Scale bar: 5  $\mu$ m. Small panels as in (C). (E) Confocal images showing endogenous syntenin-1 distribution in target T-cells forming cell-cell contacts with cells infected with VLPs. Contacts are productive sites for viral transfer and also recruit syntenin-1. Scale bar: 5  $\mu$ m. Small panels as in (C). (F) Quantification of the number of VLPs that cluster syntenin-1 at the surface of target T-cells (4°C, attached particles), in the cytoplasm (37°C, intracellular particles) or at cell-cell contacts (37°C). Results are from at least three independent experiments and more than 100 VLPs were quantified for each condition.



**FIGURE 6:** Actin polymerization is reduced in syntenin-1-depleted T-cells. (A) J77 T-cells were incubated with fluorescent VLPs (Gag-GFP) for 30 min, fixed, and stained for syntenin-1 and F-actin. The panels show projections of selected confocal planes in which a VLP was observed, with corresponding pseudocolor images below. Scale bar: 5  $\mu$ m. The zoomed image shows the particle site (boxed region in merged image) together with orthogonal sections. Quantification of VLPs that colocalized with F-actin in cells silenced or not for syntenin-1 is shown at the right. No significant difference was observed. (B) Time profile of F-actin content upon contact with HIV-1 particles, measured by flow cytometry in control or syntenin-depleted cells. Data are means  $\pm$  SD of four independent experiments. (C) F-actin accumulation at

regard, in a preliminary attempt to assess syntenin-1 partners, we have observed its association with ERMs and PI4P5K-I (Gordon-Alonso *et al.*, unpublished data). One possibility is that syntenin-1 may be able to prevent ERMs from fulfilling their role of in facilitating HIV-1 entry but without affecting CD4/CXCR4 clustering. A second option is that syntenin-1 is limiting PI4P5K-I PIP<sub>2</sub> production, and therefore, inhibiting HIV-1 entry (Barrero-Villar *et al.*, 2008). The complexity of syntenin-1 relationships deserves further investigation. Alternatively, depletion of syntenin-1 might simply allow a higher pool of free PIP<sub>2</sub> to accumulate at the plasma membrane after HIV-1 attachment, where it could mediate the recruitment of proteins, such as, ERMs, that facilitate HIV entry (Barrero-Villar *et al.*, 2008, 2009).

The fact that syntenin-1 clustering around the virus-like particle vanishes after viral entry is consistent with participation of syntenin-1 at the plasma membrane during HIV-1 entry, probably as a consequence of its association with CD4 or its recruitment through PIP<sub>2</sub>. We therefore propose a dynamic model in which syntenin-1 is first recruited to the viral attachment site through CD4 and/or PIP<sub>2</sub>. At this location, syntenin-1 would trigger F-actin polymerization via Rac GTPase, as has also been suggested for other processes mediated by this GTPase (Sala-Valdes *et al.*, 2012). This is consistent with the fact that Rac is the Rho GTPase specifically activated during HIV-1 entry (Pontow *et al.*, 2004; Harmon *et al.*, 2010). Because syntenin-1 regulates Rac-triggered F-actin polymerization through the adaptor protein M-Rip during T chemotaxis (Sala-Valdes *et al.*, 2012), it is conceivable that this or a similar signaling axis could be involved during HIV-1 entry. Indeed, syntenin-1 Tyr-4 is essential for Rac-mediated T-cell chemotaxis and superantigen presentation (Sala-Valdes *et al.*, 2012) and seems also to be crucial for syntenin-mediated inhibition of HIV infection. Subsequently, F-actin must be locally depolymerized to facilitate the nucleocapsid entry (Yoder *et al.*, 2008; Vasiliver-Shamis *et al.*, 2009), which is favored (F-actin depolymerization) in the absence of syntenin-1. In this context, PIP<sub>2</sub> induction by HIV-1 might be the signal that shuts down local F-actin polymerization while activating F-actin clearance.

In summary, our results have unveiled a critical regulatory role for syntenin-1 in controlling HIV-1 entry into target T-cells through altering actin polymerization, most likely in a PIP<sub>2</sub>-regulated manner.

## MATERIALS AND METHODS

### Cell lines and reagents

The Jurkat-derived human T-cell line J77 Vβ8<sup>+</sup> J77cl20 was grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS; Cambrex Bioscience, Charles City, IA). The CEM-T4 human T-lymphoblast-like cell line (courtesy of Paul Clapman), and the HxBc2 Jurkat-derived T-cell line (courtesy of Joseph Sodrowski) were obtained from the National Institutes of Health (NIH) AIDS

Research and Reference Reagent Program and were cultured according to NIH instructions. The chronically infected MT4-NL4.3-GFP cell line (MT4-HIV-GFP) was generated by Nuria Izquierdo-Useros at the laboratory of Martinez-Picado (IrsiCaixa, Barcelona, Spain).

The biotinylated monoclonal anti-CXCR4 antibody was from BD PharMingen. Mouse monoclonal anti-α-tubulin was from Sigma-Aldrich (Saint Louis, MO). Anti-CD4 monoclonal antibodies used were HP2/6 (Gordon-Alonso *et al.*, 2006), OKT4 (eBioscience), RPA-T4 (eBioscience, San Diego, CA), CD4v4-FITC (BD PharMingen, Franklin Lakes, NJ), and MEM 241 (Abcam, Cambridge, UK). Rabbit polyclonal anti-syntenin-1 was from Synaptic Systems (Göttingen, Germany). The anti-CD45 mAb was clone D3/9, previously described (Gordon-Alonso *et al.*, 2006). Anti-PIP<sub>2</sub> mAb was from Santa Cruz Biotechnology (clone 2C11; Santa Cruz, CA). Anti-GFP antibodies (pAb for IP and mAb for WB) were from Living colors (Clontech, Mountain View, CA). HRP-conjugated secondary antibodies were from Pierce (Rockford, IL).

The intracellular fluorescent trackers CMAC, Calcein-AM, and CM-TMR and all fluorochrome-conjugated secondary antibodies and phalloidins were from Molecular Probes (Camarillo, CA). The HIV-1-specific fusion inhibitor T20 (Enfuvirtide) was from Roche Diagnostics.

### Cell transfection, DNA and siRNA

J77 cells ( $2 \times 10^7$ ) were electroporated in cold Optimem (Life Technologies-BRL, Camarillo, CA) with DNA (20 mg) or with siRNA (1.25 mM) using a Bio-Rad GenePulser II electroporator (240 V; 950°F). Fluorescent protein expression and siRNA knockdown were tested respectively by flow cytometry (24 h) and Western blot analysis (48 h).

The syntenin-1 GFP fusion proteins Psrα-HAGFP-syntenin-1 and syntenin-1-Y1-GFP have been described previously (Sala-Valdes *et al.*, 2012). PIP<sub>2</sub>-enriched plasma membranes were monitored during Env-mediated membrane fusion or VLP attachment by transfecting cells with the biosensor PLCδ-PH-GFP (Varnai and Balla, 1998). GST-syntenin-1-HA and GST-syntenin-1-Myc were kindly donated by F. Lozano (Hospital Clinic, Barcelona, Spain).

Negative control siRNA was from Eurogentec (Serain, Belgium) and the specific siRNAs against syntenin-1 were from Thermo Scientific Dharmacon (oligonucleotides J-008270-08 and J-008270-06).

### Preparation and production of HIV-1 and virus attachment and entry assay

Preparation of HIV-1 NL4.3 and measurement of viral replication were as previously described (Valenzuela-Fernandez *et al.*, 2005). Gag-GFP and Gag-Cherry fluorescent VLPs were produced as previously described (Izquierdo-Useros *et al.*, 2009) by cotransfecting 293T cells with the HIV Gag-eGFP/Cherry plasmids and the envelope plasmid pHXB2.

contacts between infected MT4-HIV-GFP cells and silenced or control target cells was quantified using the synapse-measure plug-in of Image J. The medians and interquartile ranges are shown. More than 100 conjugates were counted for each condition in two independent experiments ( $p = 4.08 \times 10^{-31}$ ). (D) MT4-HIV-GFP infected T-cells were incubated with control or syntenin-depleted target T-cells for 2 h, fixed, and stained for PIP<sub>2</sub> with anti-PIP<sub>2</sub> mAb. Quantified PIP<sub>2</sub> labeling at Env-mediated cell contacts is presented as the percentage of PIP<sub>2</sub> accumulated at cell contacts. More than 100 conjugates were counted for each condition ( $p = 1.1 \times 10^{-5}$ ). (E) Env+ HxBc2 T-cells were incubated with control or syntenin-depleted target T-cells for 2 h and then fixed, and the conjugates were stained for PIP<sub>2</sub> with anti-PIP<sub>2</sub> mAb. Quantified PIP<sub>2</sub> labeling at cell contacts is presented as the percentage of PIP<sub>2</sub> accumulated at heterotypic Env-driven cell-cell contacts and at homotypic Env-independent contacts (between two target T-cells). More than 100 conjugates were counted for each condition ( $p = 2.1 \times 10^{-4}$ ). (F) Control or syntenin-depleted cells expressing PLCδ-PH-GFP were incubated with fluorescent VLPs (Gag-Cherry) for 30 min, fixed, and analyzed by confocal microscopy. The bar chart shows quantification of PIP<sub>2</sub> enrichment at VLP contacts, presented as the percentage of PIP<sub>2</sub> recruited to VLP-cell contacts. More than 100 VLP contacts were counted for each condition ( $p = 0.038$ ). The right panel shows a sample confocal image in which several VLPs are attached to one pole of the target T-cell (arrows). Scale bar: 5 μm.

For p24 production, T-cells were infected with 100 ng of HIV-1 NL4.3 per million cells for 2 h at 37°C (which is equivalent to 1–2 multiplicity of infection or viral particles per cell), and then extensively washed with medium to remove nonattached viral particles. Infected T-cells were kept in culture for 3 d, at which time supernatants were harvested and p24 concentration measured by ELISA (Innotest HIV-1 antigen mAb; Innogenetic, Ghent, Belgium).

For HIV attachment and entry measurements, T-cells were infected with 20 ng of HIV-1 NL4.3 per million cells for 2 h at 4°C (attachment) or 37°C (attachment + entry), and then extensively washed to remove viral input and lysed with RIPA buffer (50 mM Tris HCl, pH 8, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS). p24 was measured by ELISA (Innotest HIV-1 antigen mAb; Innogenetic).

### Luciferase virus assay

Replication-deficient luciferase-HIV-1 particles were kindly provided by Suryaram Gummuru (Boston University, Boston, MA). Replication-deficient viral particles were produced by cotransfecting 293T cells with the luciferase-expressing reporter virus, HIV/ $\Delta$ nef/ $\Delta$ env/luc<sup>+</sup>, which contains the luciferase gene inserted into the *nef* open reading frame and does not express *env* glycoprotein (Yamashita and Emerman, 2004), and a CXCR4-tropic (Lai) *env* glycoprotein or non-T-tropic VSV glycoprotein. Virus stocks were generated by PolyFect transient cotransfection of HEK293T cells (Gummuru *et al.*, 2002). Two days after transfection, cell-free virus-containing supernatants were clarified of cell debris and concentrated by centrifugation (16,000  $\times$  g, 1 h at 4°C) and stored at –80°C until required. HIV-1 virus preparations were titrated by p24-ELISA. T-cells were infected with 200 ng of luciferase-based virus with X4-tropic HIV-1 envelope or VSV envelope per million cells for 2 h. Cells were washed extensively and luciferase activity was measured at 48 h after infection with a luciferase assay kit (Promega Corporation, Madison, WI) on a 1450 Microbeta Luminescence Counter (Wallac, Trilux).

### Env-induced cell fusion assay

A dual-fluorescence cell-fusion assay was performed as described previously (Gordon-Alonso *et al.*, 2006). Briefly, CMTMR-loaded Env<sup>+</sup> Jurkat-Hxhc2 cells were mixed with calcein-AM-loaded parental or transfected CEM-T4 cells. Fluorescence events were detected 14 h later by flow cytometry. Fusion was quantified as the ratio of double-positive events to single-positive target cells.

### Immunoprecipitation

T lymphoblasts and CEM T-cells were lysed in 1% NP-40, 1 mM MgCl<sub>2</sub>, 1 mM CaCl<sub>2</sub> in Tris-buffered saline (TBS) supplemented with protease and phosphatase inhibitor cocktails. J77 T-cells (4  $\times$  10<sup>7</sup>) were lysed in 1% NP-40 in TBS supplemented with a protease inhibitor cocktail. Lysates were centrifuged at 11,000 rpm for 10 min at 4°C, and cell lysate supernatants were incubated for 2 h at 4°C with cyanogen bromide (CNBr) beads (Amersham, Uppsala, Sweden) blocked with bovine serum albumin (BSA). Supernatants were then incubated with the indicated antibody covalently coupled to CNBr beads for 2 h at 4°C. Pellets were washed with lysis buffer, resuspended in reducing Laemmli buffer, and processed for Western blotting with the indicated antibodies.

### Immunofluorescence and confocal microscopy

T-cells incubated with viral particles, Env<sup>+</sup> cells (Hxhc2), or HIV-1 infected cells (MT4-HIV-GFP) were seeded on 50  $\mu$ g/ml poly-L-lysine for 30 min and fixed with 3% paraformaldehyde. For staining of intracellular proteins (endogenous syntenin-1, F-actin, and PIP<sub>2</sub>), sam-

ples were permeabilized for 5 min with 0.5% Triton X-100 in TBS, stained and mounted in Prolong (Invitrogen, Camarillo, CA). Images were obtained with a photomicroscope (DMR; Leica, Germany) fitted with an HCX PL APO 63 $\times$ /1.32–210.6 oil-immersion objective (Leica; Germany) and coupled to a COHU 4912–5010 charge-coupled camera.

Confocal images were obtained with a Leica TCS-SP5 confocal scanning laser microscope fitted with either an HCX PL APO lambda blue 63 $\times$ /1.4 or an HCX PL APO lambda blue 100 $\times$ /1.4 oil-immersion objective and analyzed with Image J. For clustering quantification, staining intensities were transformed with the pseudocolor-intensity look-up-table (LUT). Yellow-white patches at VLP locations were considered positive for clustering. For analyzing F-actin accumulation at cell–cell contacts, Image J software was used as previously described (Calabia-Linares *et al.*, 2011).

### Western blotting

J77 T-cells were lysed in 1% NP-40 in TBS supplemented with a cocktail of protease inhibitors and phosphatase inhibitors. Lysates were centrifuged at 11,000 rpm for 10 min at 4°C, and supernatants were mixed with reducing Laemmli buffer and boiled for 5 min. Lysates were separated by SDS–PAGE and immunoblotted with specific antibodies. Protein bands were analyzed using the LAS-1000 CCD system and Image Gauge 3.4 software (Fuji Photo Film, Tokyo, Japan).

### Flow cytometry

Cells were incubated with TBS 5% BSA for 30 min at 4°C and then with primary monoclonal antibody (30 min at 4°C). After being washed, cells were incubated with a fluorescein isothiocyanate (FITC)-conjugated secondary antibody and analyzed in a FACScalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ). Data were analyzed with Cell-Quest Pro (Becton Dickinson).

### F-actin quantification

Cells were stimulated with HIV NL4.3 free virions (100 ng/million cells), fixed with 4% formaldehyde at different time points, permeabilized with TBS 0.5% Triton X-100 (5 min), and stained with Phalloidin-Alexa647 (Molecular Probes, Camarillo, CA). Mean fluorescence intensity of F-actin staining was analyzed on a FACScalibur cytometer (BD Biosciences, Franklin Lakes, NJ).

### Statistical analysis

Statistical significance was calculated using Student's *t* test or the parametric one-way analysis of variance with Bonferroni's post hoc multiple-comparison test. Significant differences are labeled (\*, *p* < 0.05; \*\*, *p* < 0.01; \*\*\*, *p* < 0.001).

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## ARTICLE

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# ISGylation controls exosome secretion by promoting lysosomal degradation of MVB proteins

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Exosomes are vesicles secreted to the extracellular environment through fusion with the plasma membrane of specific endosomes called multivesicular bodies (MVB) and mediate cell-to-cell communication in many biological processes. Posttranslational modifications are involved in the sorting of specific proteins into exosomes. Here we identify ISGylation as a ubiquitin-like modification that controls exosome release. ISGylation induction decreases MVB numbers and impairs exosome secretion. Using ISG15-knockout mice and mice expressing the enzymatically inactive form of the de-ISGylase USP18, we demonstrate *in vitro* and *in vivo* that ISG15 conjugation regulates exosome secretion. ISG15 conjugation triggers MVB co-localization with lysosomes and promotes the aggregation and degradation of MVB proteins. Accordingly, inhibition of lysosomal function or autophagy restores exosome secretion. Specifically, ISGylation of the MVB protein TSG101 induces its aggregation and degradation, being sufficient to impair exosome secretion. These results identify ISGylation as a novel ubiquitin-like modifier in the control of exosome production.

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Exosomes are vesicles secreted to the extracellular environment by most cell types. They are key mediators of cell-to-cell communication in many different contexts, including the immune response<sup>1,2</sup> and tumour progression<sup>3,4</sup>. Exosomes originate in endosomal compartments called multivesicular bodies (MVBs), which are late endosomes containing multiple intraluminal vesicles (ILVs) formed by the invagination of the endosomal membrane. When MVBs fuse with the plasma membrane, ILVs are released as exosomes<sup>5</sup>. Alternatively, MVBs can fuse with the lysosomal compartment, resulting in degradation of their content.

Exosome composition is not a mere copy of cytosolic content; rather, specific proteins and nucleic acids are selectively sorted into exosomes. The amount and content of exosomes can moreover change in response to different stimuli<sup>6,7</sup>. Such changes in exosome composition determine the final outcome of exosome-mediated communication<sup>8,9</sup>.

The mechanisms that control exosome composition and content are still not well understood<sup>10</sup>. Posttranslational modifications such as ubiquitination may play an important role in the sorting of proteins into exosomes<sup>11–13</sup>. The endosomal sorting complex required for transport (ESCRT) recognizes ubiquitinated proteins and sorts them into ILVs<sup>14</sup>. The ESCRT complex is essential for the sorting of proteins such as epidermal growth factor receptor into MVBs that are degraded through fusion with lysosomes<sup>15</sup>, but is also involved in the regulation of exosome composition and secretion<sup>16,17</sup>. Another ubiquitin-like protein (UBL) that can modify exosomal proteins is SUMO, whose conjugation to hnRNP A2B1 is essential for the sorting of microRNAs into exosomes<sup>18</sup>, and enhances the secretion of  $\alpha$ -synuclein into extracellular vesicles (EVs) in an ESCRT-dependent manner<sup>19</sup>.

ISG15 is an interferon (IFN)- $\alpha/\beta$ -induced UBL<sup>20</sup>, which exerts its functions in two distinct states: as a free molecule (intracellular and extracellular)<sup>21</sup> or conjugated to target proteins (ISGylation)<sup>22,23</sup>. Analogous to ubiquitin, ISG15 conjugation is mediated by the consecutive action of an E1-activating enzyme (Ube1L), an E2-conjugating enzyme (UbCH8) and E3 ligases (mHERC6/hHERC5)<sup>24–26</sup>, and counteracted by the specific isopeptidase USP18 (ref. 27). ISGylation was shown to occur in a co-translational process favouring modification of viral proteins in infected cells, which, in turn, interferes with virus assembly or function<sup>28–30</sup>. Furthermore, cellular proteins involved in antiviral defense or export of viral particles have been shown to be ISGylated, supporting the antiviral function of ISG15 (ref. 28). Studies in mice have demonstrated a role for ISG15 in antiviral immunity. Hence, mice lacking ISG15 exhibit a higher susceptibility to several pathogens including virus<sup>31</sup> and bacteria<sup>32</sup>, and this is reverted in USP18-mutant mice, in which high levels of ISG15 conjugation are observed<sup>33</sup>. However, human ISG15 seems to have critical immune functions but not in antiviral immunity; unlike mice, ISG15 deficiency increased viral resistance in humans<sup>34</sup>. Specifically, free extracellular human ISG15 is important in IFN- $\gamma$ -dependent anti-mycobacterial immunity<sup>21</sup>, whereas free intracellular ISG15 is involved in USP18-mediated downregulation of IFN- $\alpha/\beta$  signalling<sup>35</sup>.

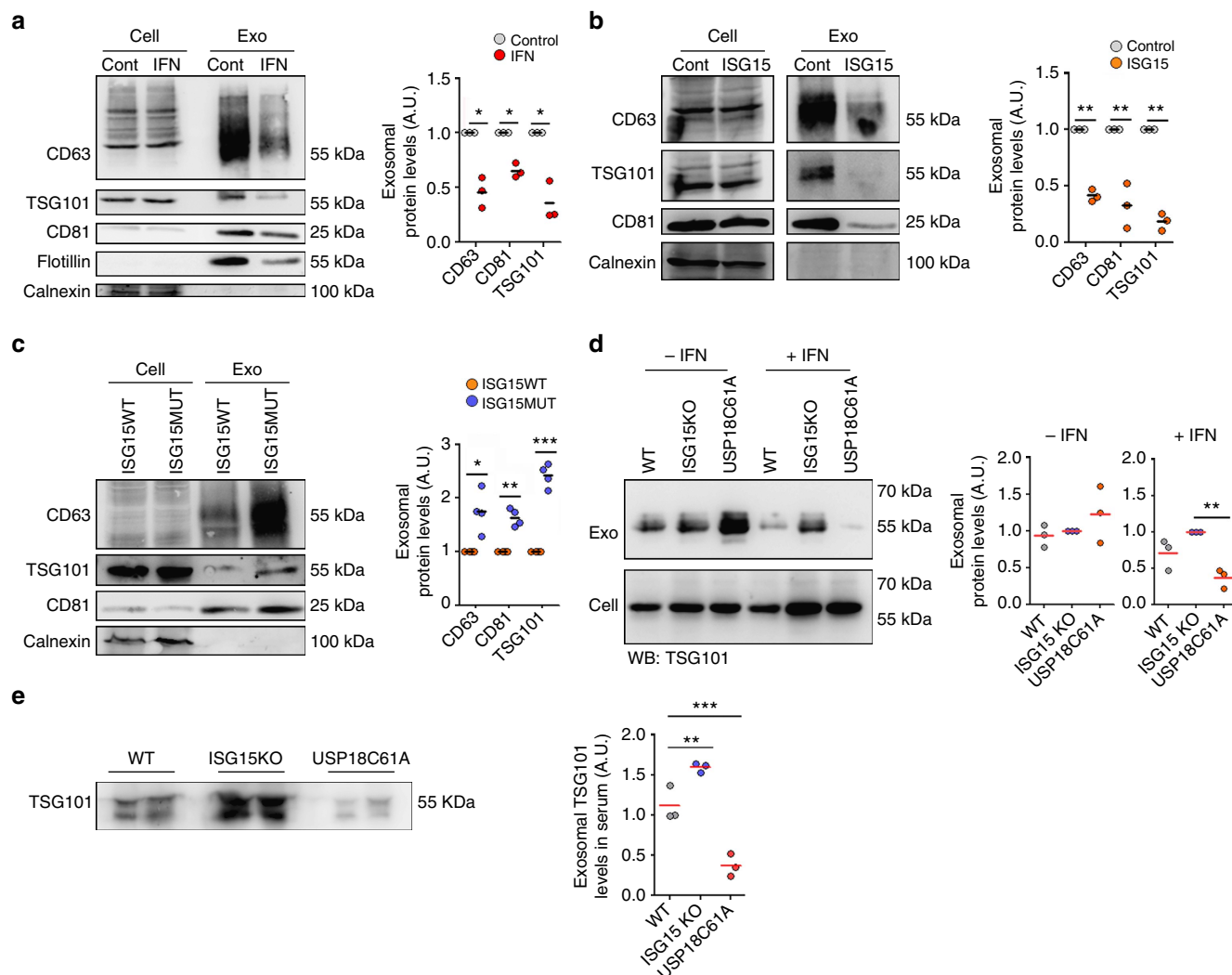
ISG15 expression blocks the process of virus-budding by different mechanisms such as the blockage of ESCRT machinery in HIV-infected cells<sup>36</sup>, or in the case of Ebola and other enveloped virus infections, inhibiting the Nedd4 E3 ubiquitin ligase<sup>37</sup>. Interestingly, exosomes and viruses share many features, and some viruses have been shown to exploit exosome and microvesicle secretion pathways<sup>38,39</sup>. In addition, exosomes are enriched in ISGylation targets, such as TSG101 (ref. 40) and heat-shock proteins<sup>41</sup>.

Here we show that IFN-I inhibits exosome secretion by inducing protein ISGylation. We demonstrate that the ISGylation of the MVB protein TSG101 induces its aggregation and degradation, and this is sufficient for impairing exosome secretion. Moreover, the ISGylation-induced defect in exosome secretion is rescued on inhibition of lysosomal function or autophagy.

## Results

**ISGylation inhibits exosome secretion.** To analyse the role of ISGylation on exosome production, we first treated Jurkat T cells with IFN-I, to induce ISG15 expression and conjugation (Supplementary Fig. 1A), and purified the secreted EVs from their culture supernatants by a serial ultracentrifugation protocol. IFN-I treatment inhibited the secretion of the classical exosome markers CD63, TSG101 and CD81 in the purified EVs (Fig. 1a). A concomitant decrease in the exosomal markers on IFN-I treatment was also observed after an additional purification step by ultracentrifugation in a sucrose gradient (Supplementary Fig. 1B). Interestingly, the few EVs secreted by IFN-treated cells were recovered in different fractions than the EVs from non-treated cells, suggesting that they might be of different nature. To further investigate the function of ISGylation in controlling EVs secretion, we next overexpressed ISG15 and the machinery responsible for ISGylation (E1, E2 and E3) in HEK293 cells, which mimics the activation of the ISG15 pathway (Supplementary Fig. 1A)<sup>24,28</sup> without activating other pathways induced by IFN-I treatment. ISGylation induction inhibited the secretion of EVs containing CD63, TSG101 and CD81 (Fig. 1b). To dissect whether the inhibition in exosomal markers secretion was due to ISG15 overexpression itself or to increased ISG15 conjugation to proteins, we overexpressed the ISGylation machinery together with an ISG15 mutated at the carboxy-terminal (ISG15MUT), thus disabling protein conjugation. Cells overexpressing ISG15MUT showed higher levels of exosomal markers in comparison with ISG15WT (Fig. 1c), demonstrating that ISG15 conjugation to proteins, but not free ISG15, is required for the inhibition of exosomal markers secretion. Nanoparticle tracking analysis (NTA) showed a decrease in the number of particles secreted on ISG15WT overexpression in comparison with ISG15MUT-expressing cells (Supplementary Fig. 1C), indicating that ISGylation is not causing a mere decrease in the sorting of exosomal markers in EVs, but an actual decrease in exosome secretion. However, NTA did not reveal any significant decrease in the secretion of nanoparticles on IFN-I treatment (Supplementary Fig. 1D).

To study the role of ISG15 in exosome secretion in a more physiological context, we analysed the secretion of exosomes by primary cells from wild-type (WT), ISG15 knockout (ISG15KO)<sup>42</sup> and USP18C61A mice, which have the ISG15 de-conjugating enzyme USP18 enzymatically inactive and thus show higher levels of protein ISGylation<sup>33</sup>. Consistent with the inhibition of exosome secretion induced by ISGylation, IFN-I treatment decreased exosome secretion in primary bone marrow-derived macrophages (BMDMs) from WT mice but not in ISG15-deficient macrophages (Fig. 1d). Furthermore, BMDMs from USP18C61A mice secrete less exosomes than either WT or ISG15KO mice on ISGylation induction by IFN-I, supporting the role of ISG15 protein conjugation in the regulation of exosome secretion. Similar results were also obtained with primary T lymphoblasts (Supplementary Fig. 1E). To assess the role of ISGylation *in vivo*, poly (I:C) was injected intraperitoneally into WT, ISG15KO and USP18C61A mice, to induce ISG15 expression<sup>43</sup>, and exosomes from blood serum analysed. Interestingly, there were less exosomes in serum from poly (I:C)-injected WT than ISG15KO mice, and even less exosomes in



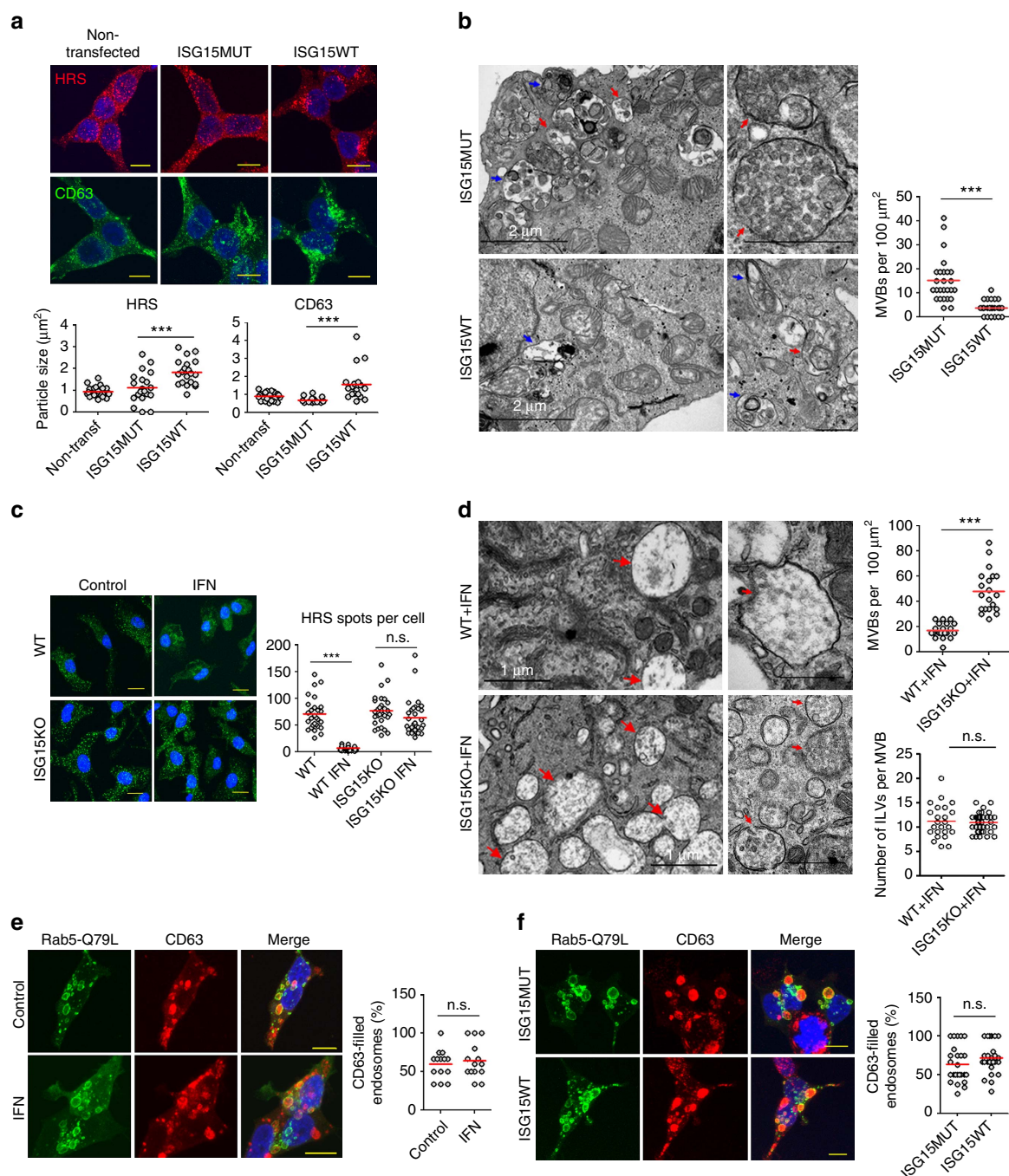
**Figure 1 | ISGylation inhibits exosome secretion.** (a) Western blot analysis of EVs purified by serial ultracentrifugation from cell culture supernatants from equal numbers of Jurkat T cells untreated (Cont) or treated with 1,000 U ml<sup>-1</sup> IFN-I for 16 h. Cells and EVs (Exo) were blotted for the exosomal markers CD63, TSG101, Flotillin and CD81, and for the endoplasmic reticulum marker Calnexin. Right graph: quantification of exosomal protein levels in the EVs obtained from IFN-I-treated and untreated cells in three independent experiments. (b) Western blot analysis of the EVs obtained from equal numbers of untransfected HEK293 cells (Cont) or co-transfected with ISG15 and the ISGylation machinery; E1, E2, E3 ligases (ISG15). Cells and EVs (Exo) were blotted for CD63, TSG101, CD81 and Calnexin. Right graph: quantification of exosomal protein levels in the EVs obtained from untransfected HEK293 cells or co-transfected with ISG15 and the ISGylation machinery in three independent experiments. (c) Western blot analysis of the EVs obtained from equal numbers of HEK293 cells co-transfected with plasmids encoding the ISGylation machinery and the functional (ISG15WT) or mutated ISG15 (ISG15MUT). Cells and EVs (Exo) were blotted for CD63, CD81 and Calnexin. Right graph: quantification of exosomal protein levels in four independent experiments. (d) Western blot analysis of the EVs obtained from equal numbers of WT, ISG15KO and USP18C61A BMDMs treated 16 h with IFN-I or left untreated. Cells and EVs were blotted for TSG101 and quantification of exosomal protein levels of IFN-I-treated and -untreated cells is shown for three independent experiments. (e) Western blot analysis of the EVs obtained in blood serum from poly(I:C)-injected WT, ISG15KO and USP18C61A mice. EVs were isolated from 250 µl of serum and blotted for TSG101. Right graphs: quantification of exosomal TSG101 protein levels of three mice per genotype; *t*-test \**P*-value < 0.05, \*\**P*-value < 0.001 and \*\*\**P*-value < 0.0001.

serum from injected USP18C61A mice (Fig. 1e), demonstrating the role of ISGylation in the inhibition of exosome secretion *in vivo*. Overall, our gain- and loss-of-function experiments strongly support the role of ISGylation in the regulation of exosome secretion *in vitro* and *in vivo*.

**ISGylation decreases MVB numbers.** To investigate the mechanisms by which ISGylation controls exosome secretion, MVBs were studied. Hepatocyte growth factor-regulated tyrosine kinase substrate (HRS) is involved in MVB formation and exosome secretion<sup>17,44</sup>, and it has been previously used as a marker

of MVBs, being present in intermediates between early and late MVBs positive for TSG101 and LBP4<sup>44,45</sup>. ISGylation induction resulted in a more perinuclear and clustered localization of HRS, as shown by the increase in the size of HRS<sup>+</sup> structures (Fig. 2a), and decreased the number of HRS<sup>+</sup> spots per cell (Supplementary Fig. 2A). Similar results were obtained with the MVB marker CD63 (Fig. 2a and Supplementary Fig. 2A), whereas the early endosome (EE) marker EEA1 did not show any apparent alteration (Supplementary Fig. 2B). Electron microscopy analyses revealed significant reduced MVBs numbers and density on ISGylation induction (Fig. 2b). In accordance, IFN-I treatment reduced HRS<sup>+</sup> spots in WT macrophages but not in ISG15KO





**Figure 2 | ISGylation decreases MVB numbers.** **(a)** Confocal microscopy analysis of the endosome markers HRS (red) and CD63 (green) in non-transfected HEK293 cells or HEK293 cells co-transfected with plasmids encoding the ISGylation machinery and the functional (ISG15WT) or mutated ISG15 (ISG15MUT). Scale bar, 10  $\mu\text{m}$ . Right graph: quantification of HRS<sup>+</sup> and CD63<sup>+</sup> average particle size per cell ( $n=20$ ). Each dot represents the average particle size from individual cells and mean is indicated in red lines. **(b)** Electron microscopy images showing representative fields with MVBs (red arrows) and lysosomes/autophagosomes (blue arrows) in HEK293 transfected as in **a**. Right graph: quantification of MVB numbers in more than 25 fields per condition. Each dot represents the number of MVBs per section and mean is indicated in red lines. Scale bar (insets), 500 nm. **(c)** Confocal microscopy analysis of HRS in WT or ISG15KO BMDMs left untreated or treated with 1,000 U ml<sup>-1</sup> IFN for 16 h. Scale bar, 10  $\mu\text{m}$ . Right graph: quantification of the number of HRS<sup>+</sup> particles per cell. Each dot represents the number of HRS<sup>+</sup> particles from individual cells and mean is indicated in red lines ( $n=28$ ). **(d)** Electron microscopy images showing MVBs (red arrows) in WT or ISG15KO BMDMs treated with 1,000 U ml<sup>-1</sup> IFN for 16 h. Upper graph, quantification of MVB numbers in 20 fields per condition. Each dot represents the number of MVBs per section and mean is indicated in red lines. Lower graph, quantification of ILV numbers per MVB. Each dot represents the number of ILVs per MVB and mean is indicated in red lines ( $n \geq 24$ ). Scale bar (insets), 500 nm. **(e)** Confocal microscopy analysis of CD63 (red) in Rab5-Q79L-GFP<sup>+</sup> endosomes (green). HEK293 cells were transfected with Rab5-Q79L-GFP and treated 16 h with 1,000 U ml<sup>-1</sup> IFN or left untreated. Scale bar, 10  $\mu\text{m}$ . Right graph: each dot represents the percentage of CD63-filled endosomes per individual cell and mean is indicated in red lines;  $t$ -test; NS:  $P$ -value > 0.05 and \*\*\* $P$ -value < 0.0001.

macrophages (Fig. 2c), and MVBs were more abundant in *ISG15KO* macrophages than in their WT counterparts on IFN-I treatment (Fig. 2d), overall suggesting a role for ISG15 in regulating MVB numbers.

To ascertain whether ISGylation decreases MVB numbers by affecting MVB formation, we transfected HEK293 cells with the constitutively active Rab5-Q79L-GFP mutant, which forms large endosomes with a mixed morphology between EE and MVBs, which facilitates the study of the first steps in MVB biogenesis<sup>46,47</sup>. ISGylation induction by either IFN-I or ISG15 overexpression did not affect the sorting of the exosomal protein CD63 into Rab5-Q79L-GFP endosomes (Fig. 2e,f). Furthermore, quantitative electron microscopy analyses did not show any significant differences in the number of ILVs per MVB on ISGylation induction (Supplementary Fig. 2C,D). To rule out any possible side effect derived from the uneven distribution of small ILVs within enlarged MVBs<sup>48</sup>, ILV numbers were also quantified in the absence of Rab5-Q79L-GFP overexpression. Accordingly, macrophages from WT and *ISG15KO* mice also showed similar levels of ILVs per MVB on ISGylation induction by IFN (Fig. 2d). Altogether, these data support that ISGylation does not impair the formation of ILVs, suggesting that it is mainly affecting later stages on the MVB pathway.

**ISGylation induces protein aggregation and degradation.** We then sought to understand how ISGylation regulates MVB numbers and exosome secretion. For this, we used ISG15 fusion proteins as models of protein ISGylation, as previously described for other posttranslational modifications such as ubiquitin or other UBLs<sup>49–51</sup>. Although fusion proteins are not exactly the same than endogenous ISGylation in lysines, in our ISG15 fusion proteins the C-terminal glycine of ISG15 is fused to the NH<sub>2</sub> group of the target protein in the N-terminal amino acid through a peptidic bond, which is chemically analogue to the isopeptidic bond formed between the endogenous ISG15 and the NH<sub>2</sub> group of a target protein lysine.

Confocal analysis showed partial co-localization of ISG15-GFP fusion protein with the MVB marker HRS and with the aggresome marker p62 (ref. 52 and Fig. 3a,b), whereas no co-localization was detected with the EE marker EEA1 (Supplementary Fig. 3A). Notably, ISGylation increased the co-localization of HRS with the lysosome marker LAMP1 (Fig. 3c), although neither LAMP1 content nor localization were affected (Supplementary Fig. 3B).

Despite its co-localization with MVB markers, green fluorescent protein (GFP) was not found in exosomes when fused to ISG15 (Fig. 3d) but rather promoted its accumulation in detergent-insoluble cell fractions (Fig. 3e), indicating that ISGylation promotes protein aggregation. Western blot analysis of cycloheximide-treated cells showed that when fused to ISG15, GFP is degraded faster than GFP alone or when fused to the ubiquitin-like modifier SUMO2, and that this degradation is delayed by the lysosome inhibitor Bafilomycin A1 but not by the proteasome inhibitor MG132 (Fig. 3f and Supplementary Fig. 3C–E), thus indicating that ISGylation of proteins promotes their degradation through the lysosome.

#### **Inhibition of MVB–lysosome fusion rescues exosome secretion.**

To assess whether ISGylation inhibits exosome secretion by inducing MVB lysosomal degradation, we studied whether inhibition of MVB fusion with lysosomes could recover exosome secretion. Bafilomycin A1 is a proton pump inhibitor that increases the pH of lysosomes and inhibits trafficking between MVB and lysosomes<sup>53,54</sup>. Bafilomycin A1 treatment recovered exosome secretion in HEK293 cells in which ISGylation was

induced by ISG15 overexpression (Fig. 4a), whereas it did not increase exosome secretion in control cells (Supplementary Fig. 4A), suggesting that ISGylation inhibits exosome secretion by re-routing MVB to lysosomes but is not impairing MVB biogenesis. This is also supported by the detection of ISGylated proteins in exosomes on secretion recovery (Supplementary Fig. 4B). Exosome secretion was also recovered despite ISGylation induction by IFN-I in Jurkat T cells when treated with Bafilomycin A1 (Supplementary Fig. 4C).

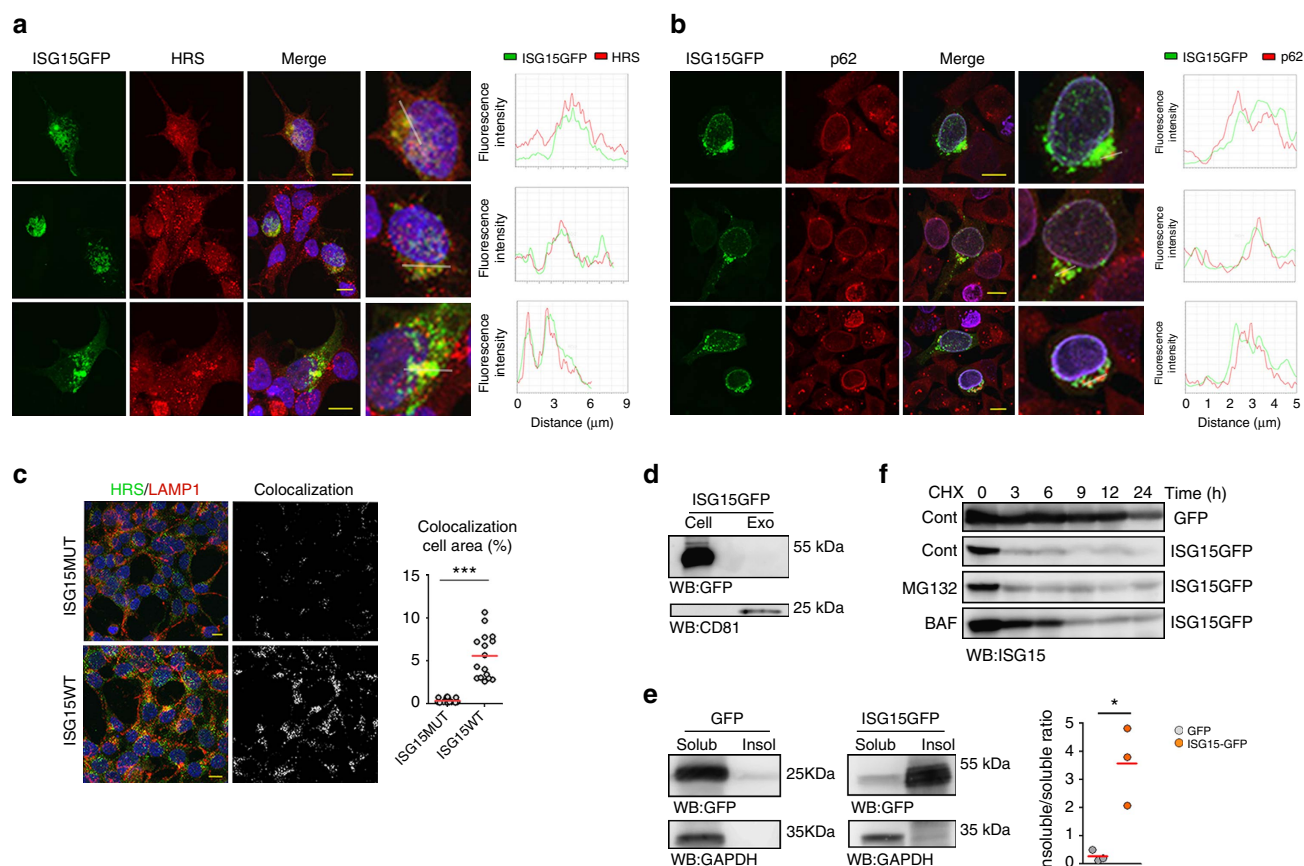
To avoid unspecific effects derived from the use of the inhibitor, we also studied exosome recovery on Rab7T22N dominant-negative mutant expression, which inhibits both endosome–lysosome and autophagosome–lysosome fusion<sup>55–58</sup>. Rab7T22N expression rescued exosome secretion in HEK293 cells despite ISGylation induction by ISG15 overexpression (Fig. 4b), whereas it did not increase exosome secretion in control cells (Supplementary Fig. 4D), supporting that the ISGylation-induced inhibition of exosome secretion is mediated by MVB fusion and degradation by the lysosome.

Fusion of MVBs with autophagosomes on induction of autophagy by rapamycin has been reported<sup>56</sup>. Interestingly, the secretion of exosomes on ISGylation induction was also recovered on silencing of the autophagy mediator ATG5 (Fig. 4c), indicating that autophagy probably contributes to the decreased exosome release induced by ISGylation. Accordingly, IFN-I treatment promoted the localization of the autophagy mediator LC3 in more acidic compartments, presumably lysosomes, supported by the loss of GFP fluorescent signal in mRFP-GFP-LC3 tandem reporter (Fig. 4d). Notably, ISGylation induction by IFN-I treatment or ISG15 overexpression did not induce general autophagy, as evidenced by unaltered levels of p62 and other autophagy markers (Supplementary Fig. 4E,F), indicating that ISGylation promotes selective autophagy and degradation of MVB without inducing a global autophagy response.

#### **ISG15 impairs exosome secretion by modifying TSG101.**

To elucidate the molecular mechanism by which ISGylation regulates exosome secretion, we focused on TSG101, a component of the ESCRT machinery involved in exosome biogenesis that has been shown to be ISGylated and to regulate the secretion of virus<sup>40</sup>. To confirm TSG101 ISGylation, we overexpressed the ISGylation machinery together with WT or mutated ISG15 fused to the V5 tag and performed V5 immunoprecipitation. Western blot analysis of TSG101 in V5-ISG15 but not in control or V5-ISG15MUT immunoprecipitates confirmed endogenous TSG101 ISGylation (Fig. 5a). Moreover, ISGylation induction by ISG15 overexpression induced endogenous TSG101 aggregation and accumulation in insoluble fractions (Fig. 5b). To address whether ISGylation of TSG101 is sufficient to inhibit exosome secretion, we generated a TSG101-GFP plasmid that mimics TSG101 ISGylation (referred as ISG15-TSG101-GG), in which ISG15 terminal glycine is conjugated to TSG101 N-terminal through a peptide bound that can be cleaved by the USP18 de-ISGylase (Supplementary Fig. 5A). Consequently, both ISGylated and de-ISGylated TSG101-GFP are detected in cells transfected with this plasmid (Fig. 5c). Interestingly, ISGylated TSG101-GFP accumulated in insoluble fractions and was degraded faster than de-ISGylated TSG101-GFP (Fig. 5d,e), consistent with ISGylation promoting TSG101 protein aggregation and degradation. The expression of the ISG15-TSG101-GG plasmid rescued exosome secretion in cells targeted for endogenous TSG101 (Fig. 5f and Supplementary Fig. 5B), probably due to the presence of a functional de-ISGylated form of TSG101. To circumvent this, we





**Figure 3 | ISG15 conjugation induces protein aggregation and degradation by lysosomes.** (a) Confocal co-localization analysis of ISG15-GFP (green) and the MVB marker HRS (red). Right graphs: fluorescence intensity profiles of ISG15-GFP (green) and HRS (red) in the regions delineated by a white line. Nuclei were stained with DAPI. Scale bar, 10  $\mu$ m. (b) Confocal co-localization analysis of ISG15-GFP (green) and p62 (red). Right graphs represent fluorescence intensity profiles of ISG15-GFP (green) and p62 (red) of the regions delineated by a white line. Nuclei were stained with DAPI. Scale bar, 10  $\mu$ m. (c) Confocal microscopy analysis of HRS (green) and LAMP1 (red) co-localization in HEK293 cells co-transfected with ISGylation machinery and functional (ISG15WT) or mutated ISG15 (ISG15MUT). Nuclei were stained with DAPI. Scale bar, 10  $\mu$ m. Co-localization area per cell was quantified by ImageJ ( $n = 16$ ). Each dot represents the percentage of co-localization area per cell surface and mean is indicated in red lines. (d) Western blot analysis of exogenous ISG15 in HEK293 cells transfected with ISG15-GFP. Cells and EVs (EXO) were blotted for GFP and CD81. (e) Western blot analysis of ISG15-GFP and GFP in 0.5% NP-40 soluble and insoluble cell fractions in HEK293 transfected with GFP or ISG15-GFP. Right graph: quantification of GFP and ISG15-GFP in the insoluble fraction respect to the soluble fraction in three independent experiments. (f) Western blot analysis of GFP and ISG15-GFP degradation kinetics in HEK293 cells transfected with GFP or ISG15-GFP and treated with cycloheximide to inhibit protein synthesis during the indicated times. Where specified, the medium was supplemented with the lysosome inhibitor Bafilomycin A1 (BAF) or the proteasome inhibitor MG132. A representative blot from two independent experiments is shown. Data from **c,e**:  $t$ -test \* $P$ -value  $< 0.05$  and \*\*\* $P$ -value  $< 0.0001$ .

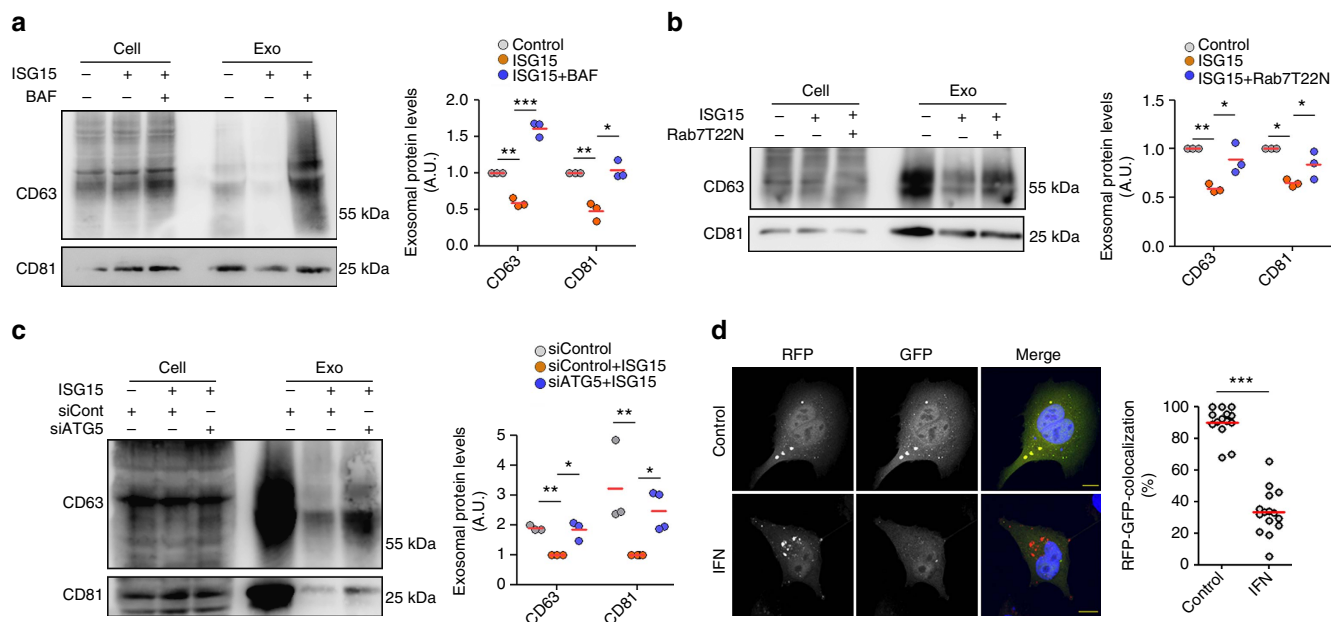
generated a non-de-ISGylable TSG101 mutant by changing ISG15 terminal glycines into alanines (ISG15-TSG101-AA), thus preventing TSG101 de-ISGylation (Fig. 5c). In cells devoid of endogenous TSG101, overexpression of the non-de-ISGylable TSG101 mutant did not restore the secretion of exosomes (Fig. 5g and Supplementary Fig. 5C), supporting that ISG15 conjugation to the ESCRT component TSG101 is sufficient to inhibit the secretion of exosomes.

## Discussion

Cells secrete a variety of EVs including shedding vesicles, coming from the direct evagination of the plasma membrane, and exosomes, coming from endosomal compartments called MVBs. The membrane of MVB invaginates to form ILVs, which are secreted to the extracellular environment on MVB fusion with the plasma membrane<sup>5</sup>. MVBs can also fuse with the lysosomal compartment for the degradation of their content. There is evidence that different types of MVB co-exist in cells<sup>59</sup>, although the differences in their composition and the mechanisms that

control their biogenesis and final fusion with the lysosome or plasma membrane are not well understood<sup>10</sup>. In addition, on specific stimuli such as starvation or rapamycin treatment, MVBs can be re-routed to promote their fusion with the lysosomal compartment and thus avoid their fusion with the plasma membrane and the concomitant secretion of exosomes<sup>56</sup> demonstrating that the final fate of MVBs is not immutable but can change under specific conditions<sup>60</sup>.

The role of the ESCRT complex in the sorting of ubiquitinated proteins into ILVs for their degradation in the lysosome is well known<sup>61</sup> and some of the ESCRT complex components are also involved in the secretion of exosomes and other EVs<sup>12,17</sup>, although their precise role in the biogenesis of the different types of EVs is not known. Other ubiquitin-like modifiers have been shown to control the composition of EVs, for example, SUMOylation of hnRNP A2B1 promotes the sorting of specific microRNAs into exosomes<sup>18</sup>, and also controls the sorting of  $\alpha$ -synuclein into EVs<sup>19</sup>. Here we identify the ubiquitin-like modifier ISG15 as a novel regulator of the exosome pathway. We show that ISGylation induction by ISG15 machinery



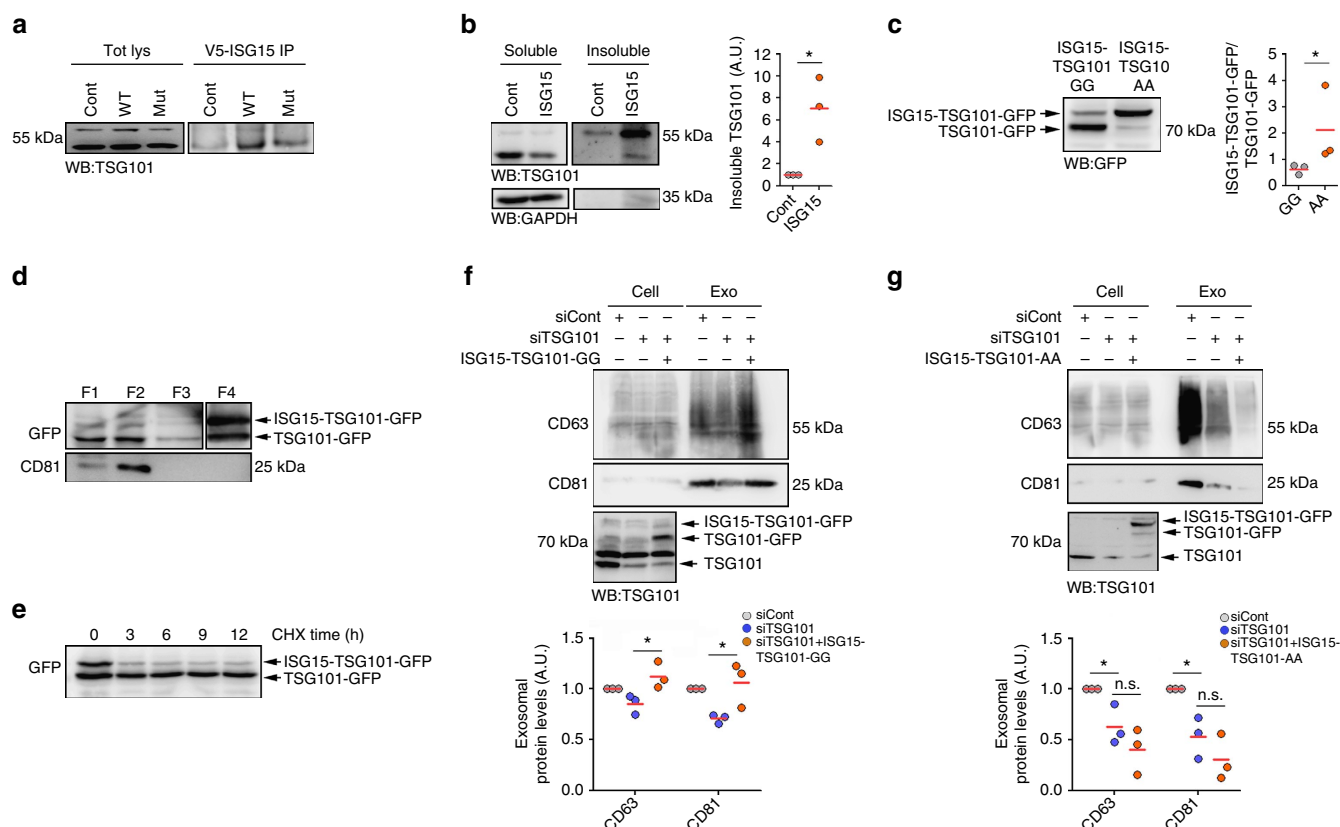
**Figure 4 | Inhibition of lysosome function upon ISGylation induction rescues exosome secretion.** (a) Western blot analysis of exosome secretion in untransfected HEK293 cells and HEK293 cells co-transfected with plasmids encoding the ISGylation machinery and ISG15, and treated with Bafilomycin A1 (BAF), when indicated. Cells and EVs (Exo) were blotted for CD63 and CD81. Right graph: quantification of exosomal protein levels in three independent experiments. (b) Western blot analysis of exosome secretion in HEK293 cells transfected with GFP or co-transfected with plasmids encoding ISG15 and the ISGylation machinery, and when indicated, with the Rab7T22N dominant-negative mutant. Cells and EVs were blotted for CD63 and CD81. Right graph: quantification of exosomal protein levels in three independent experiments. (c) Western blot analysis of exosome secretion in HEK293 cells transfected with control small interfering RNAs (siRNAs) or siRNAs targeting ATG5. After 48 h, cells were re-transfected with siRNAs together with plasmids encoding ISG15 and ISGylation machinery. Cells and EVs (Exo) were blotted for CD63 and CD81. Right: quantification of exosomal protein levels in three or four independent experiments. (d) Confocal analysis of LC3 localization into acidic compartments in HEK293 cells transfected with LC3-RFP-GFP tandem plasmid and treated 16 h with IFN-I where indicated. Nuclei were stained with DAPI. Scale bar, 10  $\mu$ m. Images were processed by ImageJ and the number of RFP<sup>+</sup> and RFP<sup>+</sup> GFP<sup>+</sup> dots quantified. Each dot represents the percentage of RFP<sup>+</sup> GFP<sup>+</sup> dots per individual cell and mean is indicated in red lines; t-test; \*\*\*P-value < 0.001. Data **a,b,c**: analysis of variance; \*P-value < 0.05, \*\*P-value < 0.001 and \*\*\*P-value < 0.0001.

overexpression or IFN-I decreases the secretion of exosomal markers. Importantly, experiments with the non-conjugable ISG15MUT and with primary cells from mice with defective function of the specific ISG15 de-ISGylase USP18 demonstrated that this effect is mediated by ISG15 conjugation to proteins and not by free ISG15. ISGylation induction by ISG15 overexpression induces a decrease in microparticle secretion, indicating that ISGylation is not merely decreasing the sorting of certain exosomal markers into ILVs but is actually decreasing exosome secretion. However, ISGylation induction by IFN-I does not induce a significant decrease in the number of secreted microparticles. This could be due to the induction of the secretion of other types of EVs by IFN-I and this is in agreement with previous reports showing that IFN-I can promote the secretion of EVs loaded with antiviral components that are transferred to other cells<sup>62</sup>. In fact, on ultracentrifugation in sucrose gradients, the few EVs secreted by IFN-treated cells are recovered in different fractions than the EVs from non-treated cells, suggesting that they might have a different nature. Alternatively, the absence of effect of IFN on global particle secretion may indicate that only the EVs derived from MVBs are affected.

We also show that ISGylation decreases the numbers of MVBs, but does not prevent the formation of ILVs. The secretion of exosomes is recovered when the fusion of MVB with lysosomes or autophagosomes is inhibited, indicating that the observed inhibition of exosome secretion is mainly mediated by the induction of MVB degradation by the lysosome. In accordance, we show that ISGylation promotes the aggregation and

degradation of proteins by the lysosome, and this is in agreement with previous reports in which ISG15-linked proteins associated with the proteins p62 and HDAC-6 involved in the autophagic process<sup>52</sup>. We have observed that ISGylation decreases the number of HRS<sup>+</sup> structures without preventing the formation of ILVs, possibly reflecting an accelerated degradation of endosomes. In addition, ISGylation increases the co-localization of the lysosome marker LAMP-1 with HRS, which is usually absent from LAMP-1-positive structures<sup>44</sup>. Whether ISGylation of MVB proteins induces the rapid fusion of MVB with lysosomes in an earlier stage of maturation, or whether it directly impairs HRS dissociation from endosomal membranes is not known.

A number of ISG15 target proteins are typically present in exosomes and MVBs<sup>41</sup>, including several components of the ESCRT complex such as TSG101, CHMP2A, CHMP4B and CHMP6 (refs 40,63,64). It is therefore conceivable that ISG15 is recruited to MVB by conjugating endosomal proteins. There, it may promote protein aggregation and enhance MVB degradation by the autophagosome-lysosome compartment, thus preventing their fusion with the plasma membrane and the secretion of exosomes (Fig. 6). We have validated the ISGylation of TSG101, which has been previously shown to control the secretion of viral particles<sup>40</sup>, and we show that TSG101 ISGylation is sufficient to impair exosome secretion. However, we cannot rule out the possibility that other endosomal proteins are being ISGylated and also contribute to the inhibition of exosome secretion induced by ISG15. TSG101 depletion has been previously shown to inhibit MVB formation<sup>65</sup> or to modify the size of MVBs and/or the number of ILVs<sup>48</sup>. However, there are significant differences



**Figure 5 | ISG15 impairs exosome secretion by modifying TSG101.** (a) Western blot analysis of TSG101 immunoprecipitation in HEK293 cells non-transfected or HEK293 cells co-transfected with plasmids encoding the ISGylation machinery and functional (ISG15WT) or mutated ISG15 (ISG15MUT). Total lysates and V5 immunoprecipitates were blotted for TSG101. (b) Western blot analysis of 0.5% NP-40 soluble and insoluble fractions from non-transfected HEK293 cells (Cont) or cells transfected with ISG15 and ISGylation machinery (ISG15). Soluble and insoluble fractions were blotted for TSG101 and GAPDH. Right graph: quantification of TSG101 protein levels in insoluble fractions in three independent experiments. (c) Western blot analysis of ISG15-TSG101-GFP ISGylation in HEK293 cells transfected with ISG15-TSG101-GFP-GG or with the non-de-ISGylable mutant ISG15-TSG101-GFP-AA. Cell lysates were blotted for GFP. Right graph: quantification of ISG15-TSG101-GFP/TSG101-GFP ratio in three independent experiments. (d) Western blot analysis of ISG15-TSG101-GFP and TSG101-GFP subcellular distribution in HEK293 cells transfected with ISG15-TSG101-GFP-GG. Fractions corresponding to cytosol (F1), membranes (F2), nucleus (F3) and insoluble aggregates (F4) were extracted and blotted for GFP and CD81. A lower exposition of the same blot is shown for F4. (e) Western blot analysis of ISG15-TSG101-GFP and TSG101-GFP degradation kinetics in HEK293 cells transfected with ISG15-TSG101-GFP-GG and treated with cycloheximide during the indicated times. Cell lysates are blotted for GFP. (f) Western blot analysis of HEK293 cells transfected with control small interfering RNAs (siRNAs) or siRNAs targeting TSG101. When indicated, cells were co-transfected with ISG15-TSG101-GFP-GG. Cells and EVs (EXO) were blotted for CD63 and CD81. Lower gel shows cell lysates blotted for TSG101 to check TSG101 silencing and exogenous overexpression. Lower graph: quantification of exosomal protein levels in three independent experiments. (g) Western blot analysis of HEK293 cells transfected with control siRNAs or siRNAs targeting TSG101. When indicated, cells were co-transfected with the non-de-ISGylable mutant ISG15-TSG101-GFP-AA. Cells and EVs (EXO) were blotted for CD63 and CD81. Lower gel shows cell lysates blotted for TSG101 to check TSG101 silencing and exogenous overexpression. Lower graph: quantification of exosomal protein levels in three independent experiments. Data **b,c**: *t*-test; NS: *P*-value > 0.05 and \**P*-value < 0.05. Data **f,g**: analysis of variance; NS: *P*-value > 0.05 and \**P*-value < 0.05.

between depletion and ISGylation of TSG101. ISG15 may be directed to endosomes in a later stage of their maturation and here modify MVB proteins such as TSG101, and promote their aggregation and subsequent degradation by the lysosome without impairing ILV biogenesis. However, we could not completely rule out any effect of ISGylation on MVB biogenesis.

ISGylation has been previously shown to exert an antiviral activity by blocking the exit of viral particles<sup>36,40,64,66</sup>. The ISGylation of multiple MVB proteins and the consequent inhibition of exosome secretion induced by IFN-I would prevent the spreading of viruses that exploit the MVB pathway for their way out the cell. In addition, exosomes play important roles in cell-to-cell communication during the immune response, tumour progression, neuron survival and many other contexts<sup>1,3,4,67</sup>. Therefore, ISGylation induction by different stimuli such as viral infection, IFN, ischaemia<sup>68</sup> or ageing<sup>69</sup> can

be an important mechanism to regulate exosome-mediated communication in many different situations.

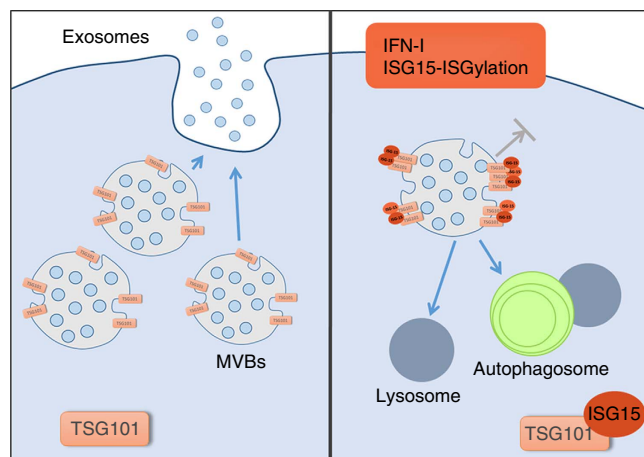
## Methods

**Cell culture.** The human Jurkat-derived T-cell line J77c20 (TCR V $\alpha$ 1.2 V $\beta$ 8) was cultured in RPMI (Sigma) containing 10% fetal bovine serum (FBS; Invitrogen). The HEK293T cell line was cultured in DMEM medium (Sigma) containing 10% FBS (Invitrogen).

Mouse primary macrophages (BMDMs) were obtained from the bone marrow of C57BL/6 WT, ISG15 knockout<sup>42</sup> and *USP18C61A* mice<sup>33</sup> (kindly provided by K. Knobloch, Freiburg University, Germany). Cells were cultured RPMI-1640 supplemented with macrophage colony-stimulating factor (30% mycoplasma-free L929 cell supernatant, NCBI Biosample accession number SAMN00155972) for 6 days, to induce cell differentiation.

Mouse primary T lymphocytes were obtained from cell suspensions prepared from spleens and peripheral lymph nodes of C57BL/6 WT, *ISG15KO USP18C61A* mice. Cells were cultured for 36–48 h in RPMI with 2  $\mu$ g ml<sup>-1</sup> concanavalin A (Sigma) and subsequently 50 U ml<sup>-1</sup> human recombinant IL-2 (Glaxo) was added





**Figure 6 | Proposed model for the role of ISGylation in the regulation of exosome secretion.** IFN-I induces ISG15 expression and conjugation to MVB proteins such as TSG101. ISGylation of MVB proteins promotes MVB fusion with lysosomes and degradation, thus inhibiting exosome secretion.

to the medium every 2 days for at least 4–6 days, to obtain differentiated T lymphoblasts.

**Exosome purification.** Cells were cultured in medium supplemented with 10% exosome-depleted FBS (bovine exosomes were removed by overnight centrifugation at 100,000 g). Supernatant fractions were collected from 16 to 20 h cell culture supernatants and exosomes were obtained by serial centrifugation as follows. Cells were pelleted ( $320 \times g$  for 10 min) and the supernatant was centrifuged at 2,000 g for 15 min, to discard debris and dead cells. The supernatant was collected and ultracentrifuged at 10,000 g for 30 min at 4 °C (Beckman Coulter Optima L-100 XP, Beckman Coulter) and exosomes were pelleted by ultracentrifugation at 100,000 g for 70 min at 4 °C. The exosome pellet was washed in PBS and collected by ultracentrifugation at 100,000 g for 70 min (Beckman Coulter Optima L-100 XP, Beckman Coulter).

**Nanoparticle tracking analysis.** Exosome numbers and size distribution were determined by measuring the rate of Brownian motion using a NanoSight LM10 system, which is equipped with fast video capture and particle-tracking software (NanoSight, Amesbury, UK). Samples were diluted before analysis to between  $2 \times 10^8$  and  $20 \times 10^8$  particles per ml, and the relative concentration was calculated according to the dilution factor. Data analysis was performed with NTA 2.1 software (Nanosight). Samples were analysed using manual shutter and gain adjustments, which resulted in shutter speeds of 15 or 30 ms, with camera gains between 280 and 560. The detection threshold was kept above 2; blur: auto; minimum expected particle size: 50 nm.

**ISGylation induction by overexpression.** HEK293 cells were co-transfected with plasmids encoding human E1, E2 and E3 ligases (kindly provided by A. García-Sastre, Mount Sinai Hospital, NYC) together with plasmids encoding WT or C-terminal mutated human ISG15 fused to the V5 tag. When indicated, cells were co-transfected with the appropriate small interfering RNAs or with the EGFP-Rab7A T22N plasmid (gift from Qing Zhong, Addgene plasmid 28048). Cells were transfected using Lipofectamine 2000 (Invitrogen). Twenty-four hours after transfection, cells were cultured in exosome-depleted medium for 20 h and exosomes were isolated from supernatants as described above. When indicated, cells were incubated overnight in exosome-depleted medium containing 20 nM Bafilomycin A1 (Merk).

**Exosome extraction from blood serum.** WT, *ISG15KO* and *USP18C61A* mice were treated with  $5 \mu\text{g g}^{-1}$  body weight poly (I:C) (InvivoGen) intraperitoneally. Blood samples were extracted from the heart 24 h after poly (I:C) injection and allowed to clot 3 h at room temperature. Blood samples were kept 2 h at 4 °C and serum was obtained by centrifugation at 400 g, 10 min at 4 °C. Cell debris were removed by centrifugation at 3,000 g, 15 min at 4 °C and serum samples were stored at  $-20^\circ\text{C}$ . Exosomes were extracted from serum as previously described<sup>70</sup>; briefly, 125  $\mu\text{l}$  of Exoquick reagent were added to 250  $\mu\text{l}$  of serum and incubated overnight at 4 °C. Samples were centrifuged at 1,500 g, 30 min and exosome pellets resuspended in RIPA buffer.

**Gene silencing.** HEK293 cells were transfected using Lipofectamine 2000 (Invitrogen) with the following small interfering RNAs: control (scrambled), ATG5 and TSG101 (SIGMA). Twenty-four hours after transfection, cells were cultured in exosome-depleted medium for 20 h and exosomes were isolated from supernatants as described above.

**Western blotting.** Cells or exosomes were lysed in RIPA buffer (50 mM Tris-HCl pH 8, 150 mM NaCl, 1% Triton X-100, 0.1% sodium deoxycholate and 0.1% SDS) containing a protease inhibitor cocktail (Complete, Roche). Proteins were separated on 10% acrylamide/bisacrylamide gels and transferred to a nitrocellulose membrane. Membranes were incubated with primary antibodies (1:1,000) and peroxidase-conjugated secondary antibodies (1:5,000), and proteins were visualized with LAS-3000. The following antibodies were used: rabbit anti-human ISG15 (Proteintech, 15981-1-AP), mouse anti-human CD63 (Calbiochem, OP171), mouse 5A6 anti-human CD81 (Santa Cruz, sc-23962), mouse anti-human TSG101 (Abcam, GTX70255), mouse anti-GFP (Living colors, 632381), rabbit anti-p62 (Sigma, P0067), mouse DM1A anti-tubulin (Sigma, F2168), rabbit anti-Calnexin (Abcam, ab10286), goat anti-mouse peroxidase (Thermo Scientific), goat anti-rabbit peroxidase (Thermo Scientific) and donkey anti-goat peroxidase (Thermo Scientific). Full immunoblots are provided in Supplementary Fig. 6.

**Fluorescence confocal microscopy.** For immunofluorescence assays, cells were plated onto slides coated with fibronectin ( $20 \mu\text{g ml}^{-1}$ ), incubated for 16 h min, fixed with 2% paraformaldehyde and stained with the indicated primary antibodies (1:100) followed by secondary antibodies (1:500). When indicated, cells were previously transfected with GFP-Rab5CA (Q79L) plasmid (gift from Sergio Grinstein, Addgene plasmid 35140) or LC3-GFP-RFP tandem construct (gift from Tamotsu Yoshimori, Addgene plasmid 21074). Samples were examined with a Leica SP5 confocal microscope (Leica) fitted with a  $\times 63$  objective and images were processed and assembled using Leica software. The following antibodies were used: anti-CD63 (clone Tea 3/18, generated in the laboratory), anti-HRS (Abcam, ab72053), anti-EE1A (Santa Cruz, sc-6415), anti-LAMP1-647 (BioLegend, 328612), anti-p62 (Sigma, P0067), goat anti-mouse-488 and goat anti-rabbit-RX (Life Technologies). Images were processed and quantified using LAS-AF and ImageJ.

**Cloning.** ISG15-GFP plasmids were generated by excision of ISG15 from the pCAGG plasmid with XhoI and EcoICRI restriction enzymes, followed by insertion between the XhoI and AfeI sites of pAcGFP-N1. ISG15 stop codons were mutated using the QuickChange Site-Directed Mutagenesis kit (Agilent). ISG15-TSG101-GFP was obtained by inserting TSG101 into ISG15-GFP plasmid with XhoI and BamHI restriction enzymes. Non-de-ISGylable mutant ISG15-TSG101-GFP-AA was obtained by mutated terminal glycines into alanines using the QuickChange Site-Directed Mutagenesis kit (Agilent). Ubiquitin-GFP expressing plasmid was obtained from Addgene (gift from Nico Dantuma, Addgene plasmid 11928).

**Protein aggregation.** HEK293 cells were transfected with GFP or ISG15-GFP plasmids using Lipofectamine 2000 (Invitrogen) and 48 h later lysed in lysis buffer (10 mM Tris-HCl pH 8, 100 mM NaCl, 1 mM EDTA, 0.5% NP40 and 50 mM iodoacetamide) supplemented with protease inhibitor cocktail (Complete, Roche). Cells were centrifuged for 5 min at 13,000 g, and the supernatant (S1) and pellet (P1) were saved. P1 was resuspended in lysis buffer containing 2% SDS, sonicated and centrifuged at 16,000 g for 10 min. The pellet (P2) was resuspended in lysis buffer containing 2% SDS and sonicated. S1 and P2 were loaded on a poly-acrylamide gel and blotted for GFP. Subcellular fractionation was performed with ProteoExtract Subcellular Proteome Extraction kit (Calbiochem).

**Protein degradation.** HEK293 cells were cultured in six-well-plates and transfected with GFP or ISG15-GFP using Lipofectamine 2000 (Invitrogen). Cells were treated with  $40 \mu\text{g ml}^{-1}$  cycloheximide (Sigma) to inhibit protein synthesis and were lysed at the indicated time points. When indicated, the medium contained 20 nM Bafilomycin A1 (Merk) or 40  $\mu\text{M}$  MG132 (Sigma).

**Immunoprecipitation.**  $1 \times 10^7$  HEK293 cells per condition were lysed (25 mM Tris pH 8, 150 mM NaCl, 2 mM  $\text{MgCl}_2$ , 0.5% NP-40 and protease inhibitors) and incubated for pre-clearing with pre-washed Protein G Dynabeads (Invitrogen; 50  $\mu\text{l}$  per condition; 1 h, 4 °C). Fifty microlitres of Dynabeads per condition were washed twice in 0.01% Tween PBS and resuspended in 200  $\mu\text{l}$  of 0.01% Tween PBS containing 5  $\mu\text{g}$  mouse anti-V5 antibody (Life Technologies) per condition and incubated 1 h at 4 °C. Pre-cleared lysates were incubated with antibody-conjugated Dynabeads (1.5 h, 4 °C). Antibody-conjugated Dynabeads were washed six times with lysis buffer and transferred to clean tubes. Protein loading buffer was added, samples were boiled at 95 °C for 5 min and processed for immunoblotting.

**Electron microscopy and MVB quantification.** Cells cultured on dishes were washed in PBS and fixed for 1 h in 2.5% glutaraldehyde in 0.1 M phosphate buffer at room temperature. Then, cells were slowly and gently scrapped and pelleted in eppendorf tubes. Pellets were washed in phosphate buffer and incubated with 1%

OsO<sub>4</sub> for 90 min at 4 °C. Then, samples were dehydrated, embedded in Spurr and sectioned using Leica ultramicrotome (Leica Microsystems). Ultrathin sections (50–70 nm) were stained with 2% uranyl acetate for 10 min and with a lead-staining solution for 5 min and observed using a transmission electron microscope, JEOL JEM-1010 fitted with a Gatan Orius SC1000 (model 832) digital camera.

For the calibration of images, quantification and analysis ImageJ was used. MVBs were identified and counted by morphology, having only discrete ILVs. Lysosomes contain multilamellar profiles. At least 20 MVBs were analysed per experiment from separate cells. Data were analysed from duplicate or triplicate separate experiments and two to four grids were used for each condition. The minimum number of cells scored for each condition was 20. Box scatter plots were generated using Prism GraphPad software and statistical tests were performed in Microsoft Excel. Data are means ± s.d. and \*\*\**P*-value < 0.0001.

**Data availability.** The data that support the conclusions of this study are available from the corresponding author upon request.

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## Author contributions

C.V.-B., F.B., I.F.-D., D.T., O.M.-G., S.B. and C.E. performed experimental work. C.V.-B., F.B., M.M., S.G. and F.S.-M. designed research. C.V.-B., F.B. and F.S.-M. analysed data and wrote the manuscript.

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# Post-translational add-ons mark the path in exosomal protein sorting

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**Abstract** Extracellular vesicles (EVs) are released by cells to the extracellular environment to mediate inter-cellular communication. Proteins, lipids, nucleic acids and metabolites shuttled in these vesicles modulate specific functions in recipient cells. The enrichment of selected sets of proteins in EVs compared with global cellular levels suggests the existence of specific sorting mechanisms to specify EV loading. Diverse post-translational modifications (PTMs) of proteins participate in the loading of specific elements into EVs. In this review, we offer a perspective on PTMs found in EVs and discuss the specific role of some PTMs, specifically Ubiquitin and Ubiquitin-like modifiers, in exosomal sorting of protein components. The understanding of these mechanisms will provide new strategies for biomedical applications. Examples include the presence of defined PTM marks on EVs as novel biomarkers for the diagnosis and prognosis of certain diseases, or the specific import of immunogenic components into EVs for vaccine generation.

**Keywords** Extracellular vesicles · Exosome · Post-translational modifications · Exosomal sorting · Ubiquitin

## Introduction

### Extracellular vesicles

Extracellular vesicles (EVs) are 50–300 nm-sized nanovesicles secreted to the extracellular milieu by a vast variety of cell types to promote inter-cellular communication on cells that capture them. Such vesicle exchange is involved in many physio-pathological situations such as tumour progression, metastasis, immunomodulation and the spreading of infections [1]. EVs have a heterogeneous nature, thus they are classified according to their origin into three groups: exosomes, shedding vesicles and apoptotic bodies [2]. Exosomes are of endosomal origin. Cells release them by fusing multivesicular bodies (MVBs)-containing intraluminal vesicles (ILVs) with the plasma membrane. Shedding vesicles form directly at the plasma membrane. Finally, apoptotic bodies are vesicles that result from apoptosis and contain cellular waste [3, 4]. EVs are membrane-covered vesicles that contain proteins, nucleic acids (DNA, mRNA and miRNAs), lipids and metabolites [5]. Proteins that are enriched in exosomes include tetraspanins (CD9, CD63, CD81), cytoskeleton components (Syntenin, Drebrin, ERM), Endosomal Sorting Complexes Required for Transport (ESCRT complex) (TSG101, ALIX), Heat-Shock proteins (HSP70 and 90), Annexins, Rab proteins, among others [6]. Exosome membranes are also different from the membrane of their producer cells, mainly enriched in ceramide, sphingolipids, glycerophospholipids and cholesterol [7, 8].

Almost every cell, prokaryotic and eukaryotic, can secrete EVs. These particles appear in many fluids, including urine, amniotic fluid, blood, bile, semen, cerebrospinal fluid, breast milk, ascites and cell cultures (reviewed in [2]). Moreover, EVs contain specific external and internal markers, which vary depending on their origin. For

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instance, CD24 is a marker for urine and amniotic fluid exosomes [9]. In addition, a single cell type can simultaneously secrete different populations of exosomes [10, 11]. For example, polarized cells can deliver different populations of exosomes through the apical and basolateral poles [12].

Regarding cargo sorting, EVs receive cargoes through both ESCRT-dependent and independent mechanisms [13, 14]. The existence of heterogeneous MVBs populations becomes apparent when different components of endosomal trafficking or the ESCRT pathway are silenced or overexpressed, and when exosomal secretion is disturbed and not all exosomal markers are affected to the same extent [10, 15]. This enables distinguishing between global markers, e.g. TSG101, ALIX or HSP70; and cell-specific markers, e.g. class II Major Histocompatibility Complex (MHC-II) [3]. EV cargo also evolves with global changes to the whole cells. For example, oxidative stress modulates MVB biogenesis, endocytosis and consequently EVs composition [12].

## Post-translational modifications

Post-translational modifications (PTMs) include a collection of add-ons that provide variability to a defined set of proteins that all cells use to enable most biological processes, adapting the functionality of those proteins by varying their interactive capability, resistance to degradation, etc. In this sense, PTMs exponentially increase the level of the proteome complexity [16]. These add-ons are molecules of diverse nature that attach to specific amino acids. Multiple amino acids can receive varied PTMs. The types of added molecule include chemical groups, carbohydrates, lipids, aminoacids, proteins and nucleotides. This process is controlled by specialized enzymes responsible for the addition or removal of each modification [17]. PTMs and their regulation constitute a cellular communication code to respond in a fast, versatile and accurate mode. The fulcrum of this review is that PTMs can direct exosome loading. Exosomes do not mirror the protein composition of their parental cells, and some proteins appear enriched in exosomes by selective mechanisms of protein cargo sorting controlled by specific PTMs [18]. The main PTMs are summarized in Table 1. These include addition of chemical groups (phosphorylation, acetylation, methylation, oxidation, hydroxylation, S-nitrosylation, sulfation or sulfonation, and others), carbohydrates [N-glycosylation, O-glycosylation, glycosylphosphatidylinositol (GPI)-anchoring], lipids (myristoylation, palmitoylation, prenylation) and nucleotides (ADP-ribosylation, PARylation). To make this large problem tractable, we focus in Ubiquitin and Ubiquitin-like modifiers (UBLs) as major controllers of EV protein loading.

## Ubiquitination and Ubiquitin-like modifiers (UBLs)

This group of PTMs represents one of the most important regulatory systems of protein location, stability and function. The C-terminal (C-t) Gly of ubiquitin and most UBLs attaches covalently mainly to the  $\epsilon$ -amino group of a Lys (K) residue of the substrate protein, forming an isopeptide bond. The conjugation of ubiquitin and UBLs requires the consecutive action of three specific enzymes: Ubl-activating enzymes (E1s), Ubl-conjugating enzymes (E2s) and Ubl-protein ligases (E3s). Specific proteases reverse this process, removing ubiquitin from target proteins [19, 20]. Ubiquitin is a highly conserved, small protein of 8.5 kDa involved in diverse functions, such as protein degradation, endocytic trafficking, signal transduction and DNA repair, among others. Mono-ubiquitination is the modification of a specific Lys in a target protein. The same protein can bear multiple mono-ubiquitinated residues simultaneously. Besides, the ubiquitin protein itself contains seven Lys (K6, 11, 27, 29, 33, 48 and 63) in its sequence. These Lys can bind to other molecules of ubiquitin, generating seven types of polymeric ubiquitin chains, constituting the so called “ubiquitin code”. Moreover, M1 chains are polymeric chains formed by ubiquitin N-terminal (N-t) Met. All these chains vary in length, ranging from two to more than ten residues. They can be homotypic if the same residue is modified during elongation as in M1, K11, 48 or 63-linked chains, or heterotypic if the same chain displays different linkages [21, 22]. Moreover, a single ubiquitin can be modified with multiple chains or UBLs (NEDD8, SUMO, ISG15), generating branched chains or mixed chains, respectively. Finally, ubiquitin can be acetylated or phosphorylated [21–25]. K48-linked ubiquitin was the first described poly-ubiquitin chain related to degradation [21, 26]. On the other hand, K63-linked chains relate to many biological processes, and K63-modified proteins play important roles in diverse cellular signalling pathways, e.g. Toll-like receptor (TLR) cascade and endosomal transport [21, 27–30]. However, the specific functions of other mixed, branched or heterogeneous chains remain almost unknown [28] (Table 1). The consensus is that proteins modified with K29 and K48-linked chains are proteasome-bound for degradation. Nevertheless, M1, K6, K11, K27 and K63 chains and mono-ubiquitination regulate different cellular processes such as DNA repair, translation, inflammation and endocytic trafficking [21, 27–33] (Table 1).

UBLs share many features with ubiquitin, including their sequence and three-dimensional structure, which is a compact  $\beta$ -grasp fold. The main members of this group are SUMO1, SUMO2-3, SUMO4, ISG15, NEDD8, ATG12, FAT10, MNSF $\beta$ , UFM1, URM and UBL5 (reviewed in [34–38]). Neural precursor cells-Expressed Developmentally Down-regulated protein 8 (NEDD8) is the closest UBL to ubiquitin. NEDD8 mainly modifies proteins from the Cullin

**Table 1** Post-translational modifications of proteins

Group	Subgroup	Molecule (donor)	Protein residues	Average mass (Da)	Structure	Adding Enzyme // Cleaving Enzyme	Ref
Chemical group	Phosphorylation	Phosphate (γ-phosphate ATP)	Ser/Thr Tyr His Asp, Arg*, Lys*	94.973		Kinases (phosphotransferases) // Phosphatases	[155]
	Acetylation	Acetate (Acetyl coenzyme A)	Lys <sup>(1,2)</sup> N-t <sup>(1,2)</sup>	43.045		Acetyl-transferases (ATs) // Deacetylases (DACs)	[156,157]
	N- Methylation	Methyl group (S-adenosylmethionine, SAM)	Arg Lys	15.035		Methyltransferases // Demethylases	[158-160]
	Oxidation	Reactive Oxygen species (ROS)					[161]
	Oxidation	Anion superoxide	Cys Met	16.000		- // Superoxide dismutases	[161]
	Hydroxylation	Hydroxyl group	Pro Asn Lys	17.007		Iron-containing monooxygenases // Pro-hydroxylase and oxygenases	[162]
		Reactive Nitrogen species (RNS)					[161]
	Tyr-Nitration	Nitro group (NO <sub>2</sub> ) (Peroxynitrites)	Tyr	46.006		- // Denitrase	[163]
	S-Nitrosylation	Nitric oxide (NO)	Cys <sup>(1,2)</sup> Met	30.006		Nitrosylases // Denitrosylases, S-Nitroglutathione reductase (GSNOR)	[164]
	Other oxidative compound: hydrogen peroxide (peroxidation) and nitrate.						[161]
	Sulfation	Sulphate (Phosphoadenosyl-phosphosulfate)	Tyr	96.064		Tyrosylprotein sulfotransferases // Sulfatase	[165]
	Amidation	Amide	C-t <sup>A</sup>	16.023		Peptidylglycine α-amid monooxygenase (PAM)	[166]
	Formylation	Formate	Met Lys	28.010		Methionyl-tRNA <sup>Met</sup> transformylase	[167,168]
	Succinylation	Succinate (Succinyl-coenzyme A)	Lys	116.073		- // Sirtuin 5	[169]
	Malonylation	Malonate (Malonyl-coenzyme A)	Lys	102.047		- // Sirtuin 5	[170]
Carbohydrates	N-Glycosylation	Monosaccharides (dolichol pyrophosphate or lipid-linked oligosaccharide, LLO)	Asn (Nxt/S)	≥ 2372.1		Oligosaccharyl transferases (OST), glucosyl transferases // glucosidases I and II, mannosidases	[171-174]
	O-Glycosylation	Monosaccharides (GlcNAc, GalNAc, fucose, glucose, mannose,...)	Ser Thr Trp* Tyr*	~ 200		O-(monosaccharide)-transferase // Hydrolases	[171,172, 174,175]
	Others: C-mannosylation and glypiation						
	GPI	Glycosyl phosphatidyl-inositol	C-t <sup>(1)</sup>	~ 1900		GPI transamidase // Phosphoinositol-specific phospholipase C	[176-178]
Lipids	S-Palmitoylation	Palmitic acid (palmitoyl coenzyme A)	Cys Ser Thr	256.424		Palmitoyl acyl transferases (PATs) // Thioesterases	[179-181]
	N-Myristoylation	Myristic acid (myristoyl coenzyme A)	N-t Gly	228.371		N-myristoyltransferase (NMT)	[179-181]
	S-Prenylation						
	Farnesylation	Farnesyl (Farnesylpyrophosphate)	Cys <sup>B</sup> (CaaX box)	222.366		Protein farnesyl transferase (FT)	[181,182]
Proteins	Geranylgeranylation	Geranylgeranyl (Geranylgeranyl-pyrophosphate)		290.483		Protein geranylgeranyl transferases (GGT I and II)	[181,182]
	Aminoacids						
	Polyglutaminylation	Glutamate (up to 20)	Internal Glu	> 147.13		Tubulin-tyrosine ligase-like (TTL) protein family // Deglutamylase	[183]
	Polyglycylation	Glycine (up to 34)	Glu	> 75.067		Glycylase // Deglycylase	[184,185]
	Other: Citrullination (conversion of Arg to citrulline by PAD enzyme)						[186,187]
	Ubiquitination						
	MonoUb	Ubiquitin	Lys N-t Thr*, Cys*, Ser*	8565		E1 activating enzyme, E2 conjugating enzyme, E3 ligase E4 // Deubiquitinases	[21,22,147]
	M1/K63	PolyUB Chain: C-t bond with N-t Met, Lys63		> 8565			[88,147]
	K6/K29/K33	PolyUB Chain: C-t bond with Lys6/Lys29/Lys33		> 8565			
	K11/K27/K48	PolyUB Chain: C-t bond with Lys11/Lys27/Lys48		> 8565			
	Ubiquitin-like proteins (UBLs)						[34-38]
	SUMOylation	SUMO1	Lys	11557		E1 (SAE), E2 (Ubc9), E3 // DeSUMOylases (SENP)	[188]
		SUMO 2-3		10871			
	ISGylation	ISG15		17888		E1 (Ubc11), E2 (UbcH8), E3 // DeISGylase (USP18)	[189]
	Neddylation	NEDD8		9072		E1 (NAE), E2 (UbcH12), E3 // Isopeptidase	[40,41]
Nucleo tides	Others UBLs: ATG12, FAT10, MNSFβ, UFM1, URM, UBL5, SUMO4						[34-38]
	Modification of Ub: Phospho-Ub and Acetyl-Ub						[21-25]
	Polyubiquitin chains: Branched chains PolyUb mixed chains (modified by SUMO, NEDD8, ISG15)						
	ADP-ribosylation						
Nucleo tides	Mono	ADP-ribose (NAD <sup>+</sup> )	Tyr Asn Lys Arg Cys	559.316		ADP-ribosyltransferases (ARTs) // ADP-ribosylhydrolases	[190,191]
	Poly (PARylation)		Glu Asp			Poly(ADP-ribose) polymerases (PARPs) // Poly (ADP-ribose) glycohydrolase (PARG)	[191]

\* Found in prokaryotes

# Rare

➡ Nucleophile or electrophile site

A Almost followed by a proline

B Cys - 5 aminoacids from the C-terminus

(1) Reversible

(2) Irreversible

Structures obtained from ChemSpider Search and share chemistry (Royal Society of Chemistry) and Protein Data Bank (PDB)

RING Ligases (CRLs) superfamily. RING E3 Ligases transfer ubiquitin directly from E2 enzymes to the substrate protein forming a complex scaffolding by Cullin. CRLs play an important role in protein ubiquitination for proteasomal degradation under the control of NEDD8 [39, 40]. NEDD8 also forms chains through one of its six Lys [41]. Small ubiquitin-related modifier (SUMO) is an UBL involved in nuclear transport and organization, DNA repair, transcription, chromatin remodelling and ribosome biogenesis. It is an essential modification to maintain cell homeostasis in stress responses and has become relevant in many diseases [42]. Interferon-stimulated gene 15 (ISG15) is induced by type I interferons (IFN-I), exposure to bacterial lipopolysaccharide (LPS), viral double-stranded RNA (dsRNA), ischemia and aging [43]. It is an essential part of the innate immune response to fight against bacterial and virus infections [44]. Besides, ISG15 is also overrepresented in various types of tumours [45]. Interestingly, ISG15 can also function as a free unconjugated form with an immunomodulatory role [46].

## Exploring the sorting of modified proteins into EVs

### Sorting mechanisms to MVBs

Exosomes originate in the endocytic pathway, whereas shedding vesicles form from direct budding of plasma membrane. Therefore, the mechanisms involved in protein secretion are different [18]. Here, we will discuss how PTMs could be involved in the sorting of proteins into EVs.

#### *ESCRT-dependent and independent sorting mechanisms into ILVs*

Presently, mechanisms of protein incorporation into the ILVs of MVBs for exosomal secretion or lysosomal degradation remain unclear. One key part of the machinery that controls the incorporation of ubiquitinated proteins into ILVs is the ESCRT complex [47, 48]. This complex includes four sub-complexes that sequentially recognize protein cargos and include them into ILVs. ESCRT-0 comprises HRS and STAM1/2 subunits, which are proteins with several distinct ubiquitin-binding domains (UBDs) that form a cargo-recognition module. HRS recognizes ubiquitinated proteins through a FYVE domain necessary to interact with phosphatidylinositol 3-phosphate (PI3-P) molecules of endosomal membranes. HRS also displays PSAP domains that connect with the ESCRT-I subunit TSG101, which also binds ubiquitinated proteins. Moreover, the PSAP domains of small integral membrane protein of the lysosome/late endosome (SIMPLE) also bind TSG101. SIMPLE has a PPxY

motif that interacts with NEDD4 to be ubiquitinated, and di-Leu and YKRL motives, which are signatures of endocytic functions [14, 49]. Mutations in these motifs impair the secretion of SIMPLE to exosomes, altering MVBs biogenesis [49]. Moreover, *Hrs* conditional knock-out (KO) in dendritic cells (DC) significantly reduces exosome release, pointing to an important role for ESCRT components in exosome secretion [50].

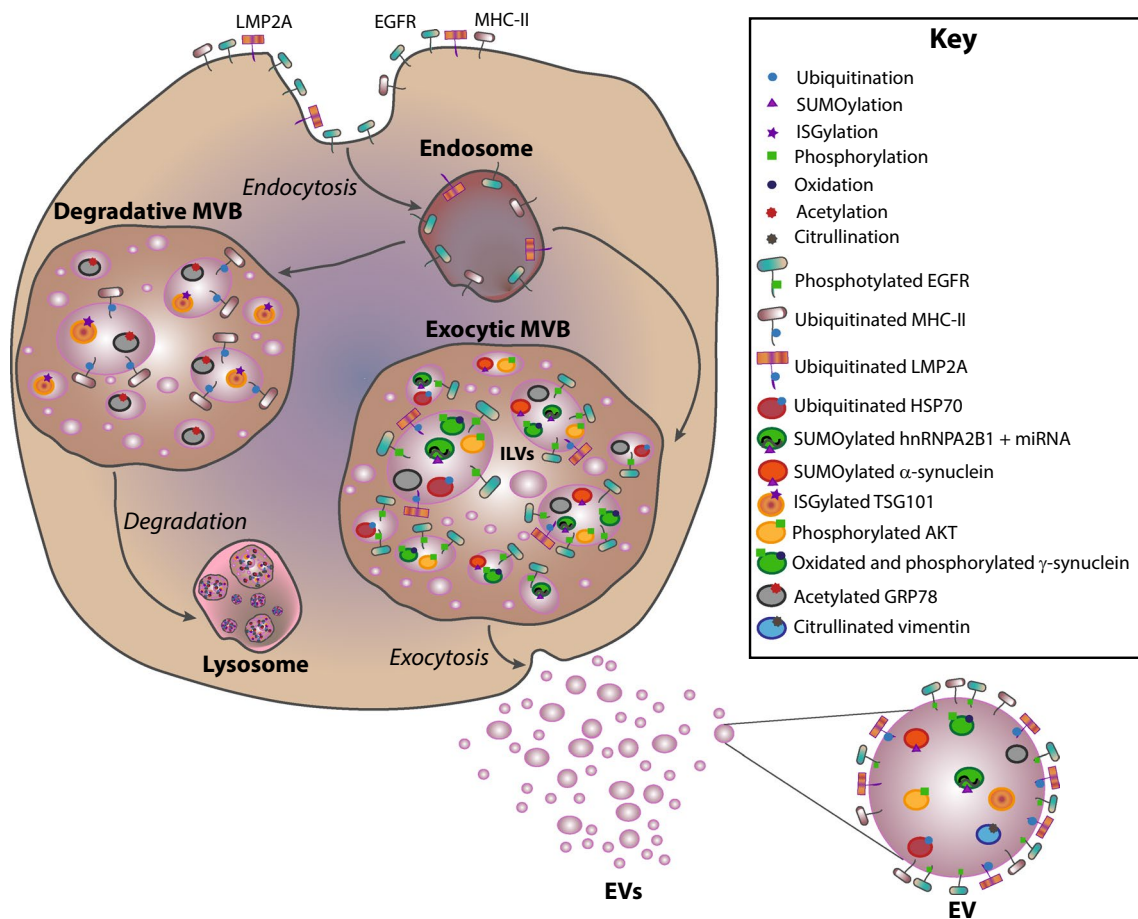
Deubiquitination is mandatory prior to packaging cargo into ILVs. To remove ubiquitin, ESCRT-III recruits deubiquitinating enzymes. ESCRT-III subunits CHMP2 A, B, CHMP3, CHMP4 A, B, C and CHMP6 bind to lipid membranes through their positively charged N-t domains, allowing high order oligomerization that results in structures over 600 kDa. Yeast ESCRT-III Snf7 (CHMP4 in humans), is not only implicated in neck-shaped oligomeric assembly for vesicle budding, but also in its scission. In this regard, the necessary energy to dissociate budding vesicles from membrane is provided by the ATPase activity of the Vacuolar protein 4 (VPS4) Complex (SKD1, CHMP5, LIP5) (reviewed in [47, 48]).

Ubiquitination of cargo proteins is necessary for recognition by ESCRT-0 components, whereas deubiquitination is a crucial step for sorting them into ILVs. However, whether ubiquitination is mandatory for driving proteins into exosomes is currently controversial. The sorting of some proteins seems independent of ubiquitination, e.g. MHCII loading into DC exosomes after T cell activation, which is associated to tetraspanin-enriched microdomains (TEMs) formed by CD9 [13, 51]. Furthermore, CD81 is important for the sorting of its associated proteins into exosomes [42]. Other studies support that the ubiquitination drives MHCII-containing MVBs to degradation upon fusion with the lysosomal system [52] (Fig. 1).

In addition, ESCRT components can be ubiquitinated themselves, for example HRS. HRS mono-ubiquitination prevents binding of ubiquitinated cargo, regulating the flow of specific proteins into MVBs [47]. The E3 ubiquitin ligase implicated in ubiquitin turnover can thus control the destiny of MVBs. For example, the activity of Mahogunin on TSG101 disrupts MVB lysosomal trafficking, whereas another E3 ubiquitin ligase called Tal abrogates MVB biogenesis [53, 54]. Associated molecule with the SH3 domain of STAM (AMSH) ubiquitin isopeptidase and ubiquitin isopeptidase Y (UBPY) removes mono-ubiquitin from HRS to recycle ESCRT components, preventing their degradation. Moreover, AMSH deubiquitinates cargoes modified with K63-linked chains, whereas UBPY recognizes both K63 and K48-linked chains. The action of these enzymes indicated a deubiquitination step that is required to sort cargoes into ILVs, and hence, into exosomes [55, 56].

However, mono and poly-ubiquitinated proteins can be found in exosomes, but not associated to the exosomal





**Fig. 1** Schematic representation of some PTMs found in EVs. Endocytosis of plasma membrane receptors (MHC-II, LMP2A and EGFR) transfer them into the endocytic pathway. Depending on the added PTMs, these receptors can have different fates. Ubiquitinated-MHC-II is preferentially directed to degradative MVBs for degradation in lysosomes, whereas, Ub-LMP2A and ph-EGFR are usually incorporated into ILVs of exocytic MVBs, which are fused with plasma membrane to be secreted as EVs [52, 77, 116]. In the case of acetylated-

GRP78 and ISGylated-TSG101, these PTMs act as cellular retention tags, promoting their degradation in lysosomes [128, 129]. However, Ub-HSP70, SUMOylated-hnRNP A2B1, SUMOylated- $\alpha$ -synuclein, ph-AKT, oxidized and phosphorylated  $\gamma$ -synuclein and citrullinated vimentin are loaded into ILVs and driven to EVs [80, 104, 106, 108, 118, 154]. Besides, unmodified TSG101 and GRP78 proteins can also be present in EVs [128, 129]. Figure key is shown at the right side. The images in the figures are not scaled

membrane [57]. Cargo deubiquitination before its packaging into ILVs seems essential, but how some ubiquitinated proteins end up in exosomes remains controversial. These contradictory results suggest that ubiquitinated proteins can evade deubiquitination; or finish up in exosomes through ESCRT-independent mechanisms. Such mechanisms do exist. In these, sorting depends on tetraspanins, sphingolipids and ceramide [58–60]. For example, LYPX(n)L motifs in the protein ALIX are implicated in its interaction with CD63 tetraspanin, and together with transmembrane proteins syndecans and syntenin induce budding and membrane abscission of ILVs [61]. In fact, an extensive study of ESCRT components downregulation revealed that of 23 ESCRT proteins studied, not all of them interfered with exosome secretion in the same way [11]. When HRS, TSG101, VPS22 and VPS24 are simultaneously silenced, MVBs are still produced and

only the formation of Epidermal Growth Factor (EGF)<sup>+</sup>-MVBs is affected, highlighting a possible mechanism of substrate selectivity [62]. Proteolipid (PLP)<sup>+</sup>-exosomes localize together with flotillin and GPI in endosomal compartments, but not with HRS. These vesicles were enriched in cholesterol and ceramide, and their secretion depended on ceramide production by the activity of sphingomyelinase 2 (nSMase2) [63]. The enzyme sphingomyelin synthase SMS2 regulates the secretion of amyloid- $\beta$  (A $\beta$ )-peptide<sup>+</sup>-exosomes. In addition, the transfer of CD63<sup>+</sup>-exosomes from T cells to APCs during immune synapse is ceramide-dependent [59, 60]. This is likely related to the fact that ceramide creates spontaneous curvature of endosomal membranes, triggering ILVs budding. Besides, other lipids metabolites such as sphingosine-1-phosphate (S1P), diacylglycerol (DAG), cholesterol, phosphatidyl choline, or

lysobisphosphatidic acid (LBPA) are involved in the MVB biogenesis and exosome secretion, although how protein cargoes are selectively transported into this kind of vesicles remains unknown [64–71].

### Ubiquitinated proteins in EVs

Ubiquitin was first observed in human urine exosomes showing a wide range of molecular weight complexes by Western blot, which suggested the presence of ubiquitinated proteins in exosomes [72]. Specific antibodies for ubiquitin and poly-ubiquitin chains allowed to identify mono-, oligo- and poly-ubiquitinated proteins in exosomes [57]. Various studies and proteomic analysis identified specific ubiquitinated proteins (reviewed in [73]) (Table 2 and Fig. 1). For example, myeloid-derived suppressor cells (MDSC) secrete exosomes enriched in ubiquitinated proteins, including endosomal trafficking proteins and histones [74]. Moreover, high molecular weight complexes of non-classical human leukocyte antigen-G (HLA-G) are ubiquitinated, but not

glycosylated, in exosomes obtained from ascitic and pleural exudates of patients [75]. Shedding vesicles also contain modified proteins such as arrestin domain-containing protein 1 (ARRDC1), which is ubiquitinated by the E3 ubiquitin ligase WW domain-containing protein 2 (WWP2). An arrestin domain in ARRDC1 anchors it to the plasma membrane, enabling its interaction with the PSAP domain of TSG101 to generate direct membrane budding [76]. In the case of ubiquitinated Epstein–Bar virus latent membrane protein 2A (LMP2A), exosome sorting depends on cholesterol, whereas the phosphorylated form was not detected in exosomes [77] (Table 2 and Fig. 1).

Phosphatase and tensin homolog deleted on chromosome 10 (PTEN) is ubiquitinated on Lys13 by the association and regulation of Nedd4 family-interacting protein 1 (Ndfip1), an adaptor protein of the Nedd4 family E3 ubiquitin ligases, appearing in this form in exosomes, although modified-Ndfip1 does not. However, its presence is necessary for the import of Nedd4, Nedd4-2 and ITCH to exosomes [14, 78]. Alanine replacement on di-Leu (LL) and YKRL motifs of SIMPLE reduces its secretion into exosomes, as well as a mutation on its PTAP motif, which controls SIMPLE binding to TSG101 for incorporation into ILVs. However, point mutations of the two PPxY motifs of SIMPLE, a binding domain for Nedd4 type E3 ubiquitin ligases, such as Nedd4 and ITCH, increase exosomal incorporation; hence SIMPLE ubiquitination does not direct its own sorting [49]. In other cases, such as Fas Ligand (FasL), ubiquitination of the proline-rich domain (PRD)-flanking Lys and phosphorylation of multiple tyrosines promote its internalization in MVBs and its secretion into EVs [79]. The COP9 signalosome (CSN)-associated protein CSN5 mediates ubiquitin isopeptidase activity. Deletion of its JAB1/MPN/Mov34 metalloenzyme (JAMM) domain increases the sorting of ubiquitinated HSP70 and HIV Gag proteins into exosomes; whereas a mutation of its associated deubiquitin activity domain promotes the enrichment of non-modified proteins in EVs [80]. These results highlight that CSN5 participates in ubiquitin-dependent and independent protein vectorization into exosomes [80] (Table 2). MARCH1, an E3 ubiquitin ligase for MHCII and CD86, is incorporated into exosomes dependent on its C-t tyrosin-based motifs [81]. This endosome sorting motif is also important for sorting of transferrin receptor into exosomes due to its interaction with ALIX [82]. Nevertheless, whether MARCH1 is ubiquitinated or not in exosomes remains unclear, although this region is important for MARCH1 auto-ubiquitination [83].

Human urine exosomes contain approximately 15% of ubiquitinated proteins. Of these, 21% are transmembrane proteins, underscoring that deubiquitination is not mandatory for the incorporation of protein cargo into MVBs and exosomes [84]. Although, ubiquitination-specific motifs remain unidentified, this PTM has preference for basic

**Table 2** Post-translational modified proteins found in EVs

Target protein	PTM	Residue	References
CryAB	Phosphorylation	Ser-59/Ser-45	[112]
Tau	Phosphorylation	Hyperphosphorylated Tau	[110]
	Phosphorylation	Thr-181/Ser-396	[117]
	Phosphorylation	-	[113]
EGFR	Phosphorylation	Tyr-1068	[116]
IRS-1	Phosphorylation	Ser-312 and Pan-Tyr	[111]
PDK1*	Phosphorylation	Ser-241	[108]
AKT*	Phosphorylation	Thr-308	[108]
SRC*	Phosphorylation	Tyr-416	[108]
ELK1 *	Phosphorylation	Ser-383	[108]
ERK 1/2*	Phosphorylation	Thr-202/Tyr-204	[108]
AMPA $\alpha$ 1 *	Phosphorylation	Ser-485	[108]
Acetyl-CoA carboxylase*	Phosphorylation	Ser-79	[108]
NCC	Phosphorylation	Thr-46, Thr-50 and Thr-55	[114]
Aquaporin 2*	Phosphorylation	Ser-256	[101]
GPCR5C*	Phosphorylation	Thr-435/Ser-395/Tyr-426	[101]
CHMP2B*	Phosphorylation	Ser-199	[101]
Fas Ligand	Phosphorylation	Tyr-7/Tyr-9/Tyr-13	[79]
	Ubiquitination	Mono-	[79]
Annexin A2	Phosphorylation	Tyr-23	[109]
$\gamma$ -synuclein	Phosphorylation	-	[118]
	Oxidation	Met-38/Tyr-39	[118]
TyA*	Myristoylation	MGCINSKRKD N-t tag	[124]
CD55	GPI-anchor	-	[125]
Vimentin*	Citrullination	-	[154]
LGALS3BP <sup>‡</sup>	Glycosylation	-	[120]
Histone H1.2*	Ubiquitination	-	[74]
HLA-G complex	Ubiquitination	-	[75]
ARRDC1	Ubiquitination	-	[76]
LMP2A	Ubiquitination	-	[77]
PTEN	Ubiquitination	Lys-13	[78]
HSP70	Ubiquitination	-	[80]
SIMPLE	Ubiquitination	-	[49]
Aquaporin-1*	Ubiquitination	Mono- and Poly-	[84]
Annexin A1*	Ubiquitination	Poly-	[84]
Plastin-3 isoform 1*	Ubiquitination	Multimono- at Lys-11 and Lys-18	[84]
HspX <sup>‡</sup>	Ubiquitination	-	[136]
GroES <sup>‡</sup>	Ubiquitination	-	[136]
GFP	Ubiquitination	Ubiquitin C-t tag	[137]
ATG85B-ESAT6	Ubiquitination	Ubiquitin C-t tag	[137]
nHer2	Ubiquitination	Ubiquitin C-t tag	[137]
GFP	SUMOylation	SUMO-1/SUMO-2 C-t tag	[106]
hnRNPA2B1	SUMOylation	-	[104]
$\alpha$ -synuclein	SUMOylation	-	[106]

\* More proteins identified in a high-throughput screening approach, proteomics [74, 84, 101, 108, 120].

<sup>‡</sup> Mycobacterial proteins detected in exosomes from human and mouse cell lines.

S More glycosylated proteins identified by lectin blotting, NP-HPLC analysis and mass spectrometry [120].

amino acids and positively charged residues upstream of the ubiquitination site, hydrophobic residues adjacent to it and additional Lys 6 amino acids upstream [84]. Besides, poly-ubiquitin chains appear following this order of enrichment: K63 > K48 > K11 > K6 > K29 > K33 > K27. This shows that K63, K48 and K11-linked chains are the most representative poly-ubiquitin topologies in exosomes [84]. Furthermore, platelet exosomes contain poly-ubiquitin chains and in higher numbers compared to platelets microparticles [85] (Table 2).

### Deciphering the ubiquitin code in EVs

The ubiquitin code comprises the combination of mono-, poly- and branched-ubiquitin chains and even other PTMs, which allow a wide range of possibilities for the regulation of proteins. K48-linked chains require at least four ubiquitin units to attach to the target protein to enable recognition by the 26S proteasome [86, 87]. In particular, the proteasome subunit S5a/Rpn10 contains an ubiquitin-interacting motif (UIM) in the hydrophobic patch of its C-t region, which enables recognition of this specific poly-ubiquitin chain. Inside the proteasome, ubiquitin molecules are removed from target proteins for recycling, whereas the rest of the proteins are cleaved into small peptides (3–25 amino acids of length). There is some evidence suggesting that other chains, e.g. M1, K6, K11, K27 and K29 could be involved in proteasomal degradation of target proteins [21, 30, 33, 86]. Moreover, a new conjugation factor, named E4, participates in the generation of efficient poly-ubiquitinated-linked chains under stress conditions to direct modified proteins to the proteasome, demonstrating the existence of an E4-dependent multi-ubiquitination pathway for degradation [88] (Fig. 2). K63-linked chains do not end up in the proteasome as they bind specifically to the ESCRT-0 complex, specifically to UIMs found in HRS and STAM1 components [27, 89]. These interactions condense proteins modified with K63-linked poly-ubiquitin chains into endosomes, promoting their import into ILVs of MVBs and possibly, their subsequent delivery into exosomes [90]. In fact, K63-linked chain is the most representative ubiquitin topology found in EVs [84] (Fig. 2).

How ubiquitin-binding proteins detect unequivocally a specific conformation of poly-ubiquitin chains may explain the selective targeting of a given protein within the cell. For example, M1 and K63-linked chains display a fairly linear or open space conformation, whereas K6, K11, and K48-linked chains create closed structures [21, 33, 91, 92] (Table 1). Open-conformation chains only limitation is that imposed by isopeptide bonds that connect them [21, 33]. However, closed conformation chains have multiple steric hindrances, which alters ubiquitin space structure by exposing and concealing different parts of ubiquitin residues, creating diverse

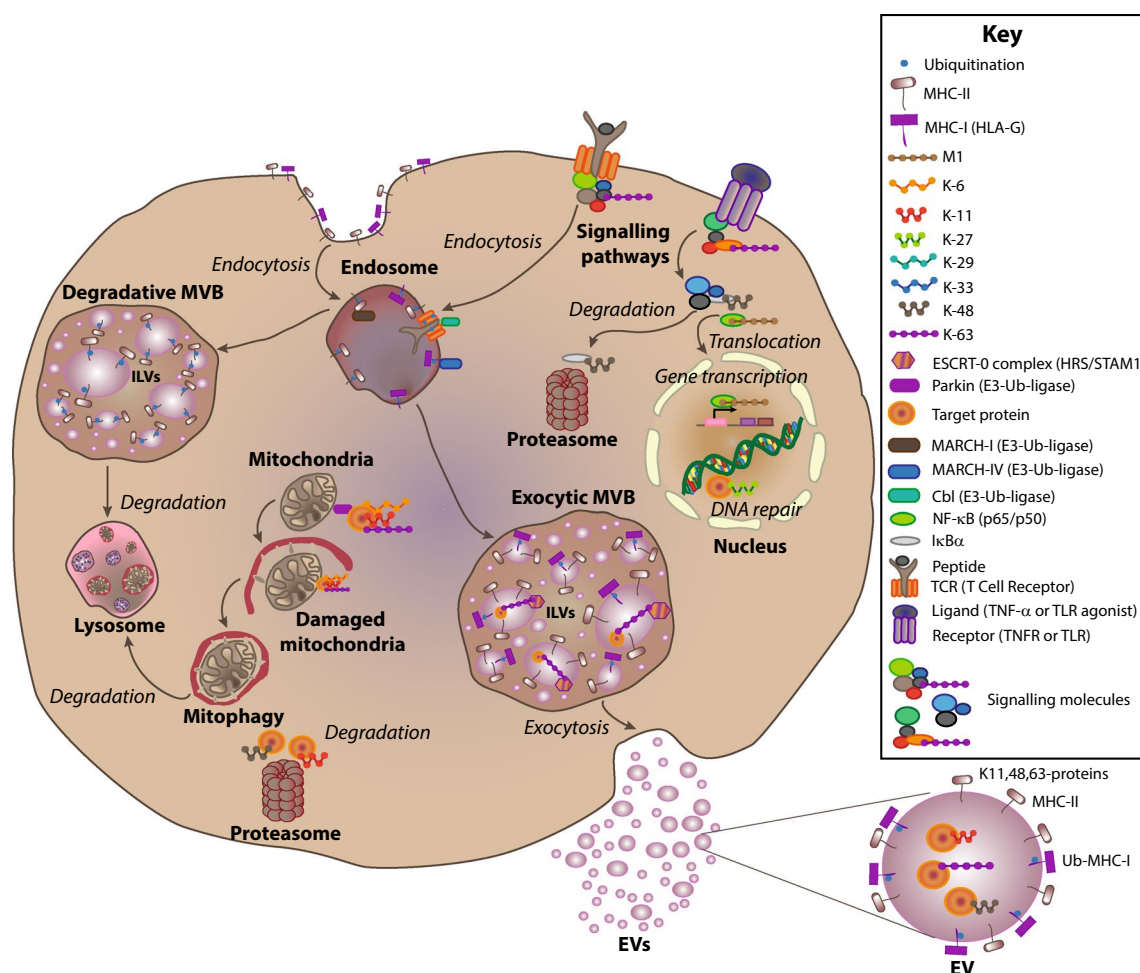
topologies intrinsic to a specific type of linkage [21, 91]. In the cell, specific proteins bind to a unique type of ubiquitin linkage topology [91]. Because distances between ubiquitin units within a chain are different in K63 or K48-linked chains, UBDs of cellular machinery display small spaces between UIMs to specifically recognize K48-chains, whereas larger spacers are used for K63 binding [91, 92]. The use of a variety of UBDs structures not only allows discriminating between open and close conformations, it also enables distinguishing among different open structures such as K63- and M1-linked chains [91, 92]. All these possibilities of ubiquitin topologies and UBDs compose a fine and selective language among ubiquitinated-target proteins and ubiquitin-interacting proteins.

In a similar way, depending on the different distances between UIMs inside UBDs, certain E3-ubiquitin ligases elongate specific types of K-linked chains associated to target proteins. Besides, the wide variety of E3-ubiquitin ligases described in eukaryotic cells and their tissue restrictions amplify the code of ubiquitin chains. For example, the Cbl family of E3-ubiquitin ligases are highly expressed in hematopoietic cells and are involved in the downregulation of many signalling pathways controlling T and B cell receptors and integrins [93, 94]. However, MARCH1 is represented in antigen presenting cells (APCs), modifying class II MHC molecules, downregulating their expression in the plasma membrane, whereas MARCH-IV regulates class I MHC molecules using a similar mechanism [95, 96]. In this regard, a possible explanation regarding how K48-linked poly-ubiquitin chains evade proteasomal degradation and travel into exosome involves the collaboration of certain ubiquitin-binding proteins located in endosomal pathway (Fig. 2).

Branched poly-ubiquitin chains display the particular signalling properties of their K48 or K63 components, although they are recognized by multiple linkage-specific enzymes [97]. This underscores the predominance of certain chain topologies within a mixed-linkage poly-ubiquitin chain. The sorting of ubiquitin chains other than K63-linked requires mechanisms different from those dependent on ESCRT components. The incorporation of SUMO-2/3 following ubiquitination creates heterologous SUMO-2/3-Ubiquitin chains responsible for enhancing the degradation of I $\kappa$ B $\alpha$  by the proteasome after the activation of the Tumour Necrosis  $\alpha$  (TNF $\alpha$ ) signalling pathway [23]. In stress situations, NEDD8 can also modify ubiquitin Lys48 [25]. A recent study described how ISGylation of ubiquitin Lys29 negatively controls the turnover of ubiquitinated proteins [24].

Interestingly, ubiquitin can be phosphorylated on Ser65 by PTEN induced putative kinase 1 (PINK1), enhancing its interaction with the mitochondrial E3 ubiquitin ligase parkin [98]. This phosphorylation-dependent mechanism increases the enzymatic activity of parkin, decreasing the





**Fig. 2** The ubiquitin code in cellular functions. MHC-I and II and TCR are preferentially retained in endosomes by the action of specific E3-Ub-ligases MARCH-IV, I and Cbl, respectively. Whereas Ub-MHC-II is mainly degraded by lysosomes, Ub-MHC-I (HLA-G) can be sorted into EVs. Signalling proteins downstream of TCR, TNFR or TLR can be modified by K63-linked chains to mediate signal transduction or by K48-linked ones, such as IκBα to be degraded in the proteasome. M1-linked chain is added to NF-κB to allow its nuclear translocation to start gene transcription. K27-linked chains are related with DNA repair processes, while K6, K11 and K63

chains can modify mitochondrial proteins to initiate mitophagy of damaged mitochondria by the E3-Ub-ligase Parkin. Proteins with K11 and K48-chains are principally transported to the proteasome to be degraded. Most of the proteins modified with K63-linked chains interact with ESCRT-0 and are vectorised in ILVs of exocytic MVBs to be delivered into EVs. Although Ub-MHC-II is mainly degraded, unmodified MHC-II and Ub-MHC-I (HLA-G) are usually incorporated in exosome membrane. Besides, K11, K48 and K63-linked chains can be found in EVs. Figure key is shown at the right side. The images in the figures are not scaled

mitochondrial membrane potential [98]. Moreover, ubiquitin becomes acetylated in Lys6 and Lys48 without affecting substrate mono-ubiquitination of histone H2B [99]. However, both acetylations inhibit K11, K48 and K63-linked poly-ubiquitin chain elongation, providing a novel regulatory step of control of mono- and poly-ubiquitination [99].

Because many PTMs can modify proteins in the same residue, competition and crosstalk between different PTMs depending on different cell factors can change protein function and localization [100]. In summary, deciphering the sophisticated ubiquitin code is necessary to unveil specific functions of every chain topology in the selective import of proteins into exosomes.

### Other PTMs in EVs

Proteomic studies have contributed extensively to define the protein composition of EVs [72, 84, 101–103]. Heterogeneous nuclear ribonucleoprotein A2/B1 (hnRNPA2B1) is present in exosomes in its sumoylated form, and that is necessary for the sorting of EXOmotif-containing microRNAs into exosomes [104]. On the other hand, a study suggested that SUMO-1 could modulate the toxicity of α-synuclein in neurodegenerative disorders such as Parkinson's disease (PD) [105]. Besides, EVs can contribute to disseminate neurotoxic proteins, which favours the development of PD pathogenesis. Hence,



sumoylated  $\alpha$ -synuclein is incorporated into exosomes by a mechanism dependent on the ESCRT complex and phospholipids [106] (Table 2 and Fig. 1).

Nevertheless, ubiquitin and UBLs are not the only PTMs detected in EVs [73, 107]. For example, many phosphorylated proteins appear in exosomes, including the calcium-dependent phospholipid-binding protein Annexin A2, which is enriched in cholesterol-rich lipid raft micro-domains of exosome membranes [101, 108, 109]. The localization of this protein depends on the phosphorylation of Tyr23, which prevents its endosomal degradation and allows its incorporation into exosomal membranes [109]. Other phosphorylated proteins found in exosomes include Tau, type 1 insulin receptor substrate (IRS-1), Crystalline alphaB (CryAB), Epidermal Growth Factor Receptor (EGFR),  $\text{Na}^+\text{-Cl}^-$  co-transporter (NCC), Aquaporin 2, FasL and proteins implicated in cellular processes such as apoptosis, survival and metabolism [79, 101, 108, 110–117] (Table 2 and Fig. 1).

$\gamma$ -Synuclein acquires features of prion-like proteins by oxidation, which triggers the aggregation of other proteins, e.g.  $\alpha$ -synuclein [118]. The most frequent oxidized residues of  $\gamma$ -synuclein are Met38 and Tyr39. Oxidized  $\gamma$ -synuclein is released into exosomes and incorporated in neighbouring cells, amplifying the toxic protein-aggregation cascade that finally causes a degenerative synucleinopathy [118]. Besides, other oxidized proteins appear in EVs as described in other studies (reviewed in [107]) (Table 2 and Fig. 1). Moreover, several studies have revealed the presence of glycosylated proteins in EVs (reviewed in [73, 107]). Notably, EVs contain mainly mannose or sialic acid enriched-glycans and N-linked glycoproteins. In particular, exosomes contain large amounts of the sialoglycoprotein galectin-3-binding protein (LGALS3BP) [119–122] (Table 2).

Additionally, plasma membrane anchors induce vesicle budding directly from the plasma membrane of highly oligomeric proteins, such as the HIV Gag protein and the yeast cytoplasmic protein Tya [123, 124]. An evaluation of different membrane anchors for Tya demonstrated that myristoylation was the most effective enhancer of the formation of shedding vesicles, whereas other types such as, phosphatidylinositol-(4,5)-bisphosphate and phosphatidylinositol-(3,4,5)-trisphosphate-binding domains, prenylation/palmitoylation tag or the type-1 plasma membrane protein CD43 did not cause this effect [124]. This illustrates the role of protein anchors for the induction of shedding vesicles, which is similar to retrovirus budding [124] (Table 2). Besides, CD55 or decay-accelerating factor (DAF) anchored to a GPI molecules is selectively secreted to EVs [125].

### *The role of UBL and other PTMs in the sorting of proteins to EVs*

The function of PTMs such as oxidation, phosphorylation, glycosylation and citrullination in the incorporation of proteins into EVs, has been recently reviewed in [107]. Briefly, these PTMs can regulate EVs release and uptake, acting as a disposal mechanism for cellular harmful components or as a repair mechanism in physio-pathological conditions [107]. Similar to ubiquitin, the UBL SUMO also plays an important role in protein sorting to EVs. For example, SUMOylation is necessary for  $\alpha$ -synuclein sorting into exosomes in an ESCRT-dependent manner [106] (Table 2). A recent study showed that other UBL called autophagy-related protein 12 (ATG12) and its E3 ligase ATG3 interact with ALIX, regulating diverse functions carried out by ALIX, for example, viral budding and MVBs biogenesis [126]. This observation suggests that ATG12 could also participate in exosome biogenesis. Besides, isoprenylation and palmitoylation may also participate in protein delivery into exosomes, as in the case of the CINCKVL palmitoylation and isoprenylation motif, which is present in human Ras Homolog Family Member B (RhoB), determines its incorporation into ILVs in a cholesterol-dependent way and consequently in exosomes [127]. Furthermore, the expression of reporter proteins containing this palmitoylation/isoprenylation motif is enough to define a late-endosomal fate for these proteins [127].

On the contrary, ISG15, which is recognized as an immune alarm signal by cells, has an opposite role in protein sorting to exosomes. A recent study showed that ISGylation tags proteins for degradation, thereby avoiding exosome secretion and spreading [43, 128] (Fig. 1). Besides, acetylation can abrogate the sorting of proteins into exosomes. High glucose-regulated protein 78 (GRP78) is expressed and secreted into exosomes by colon cancer cells to prepare the tumor microenvironment [129]. Its secretion in exosomes is impaired by chemical inhibitors of histone deacetylases (HDACs) and silencing of HDAC6 protein, which causes the dissociation of GRP78 from HDAC6 and promotes GRP78 aggregation and interaction with VPS34 complex [129]. The use of an acetylation mimetic mutant of GRP78 (K633Q) also impairs exosome sorting and blocks tumour cell growth in vivo [129] (Fig. 1).

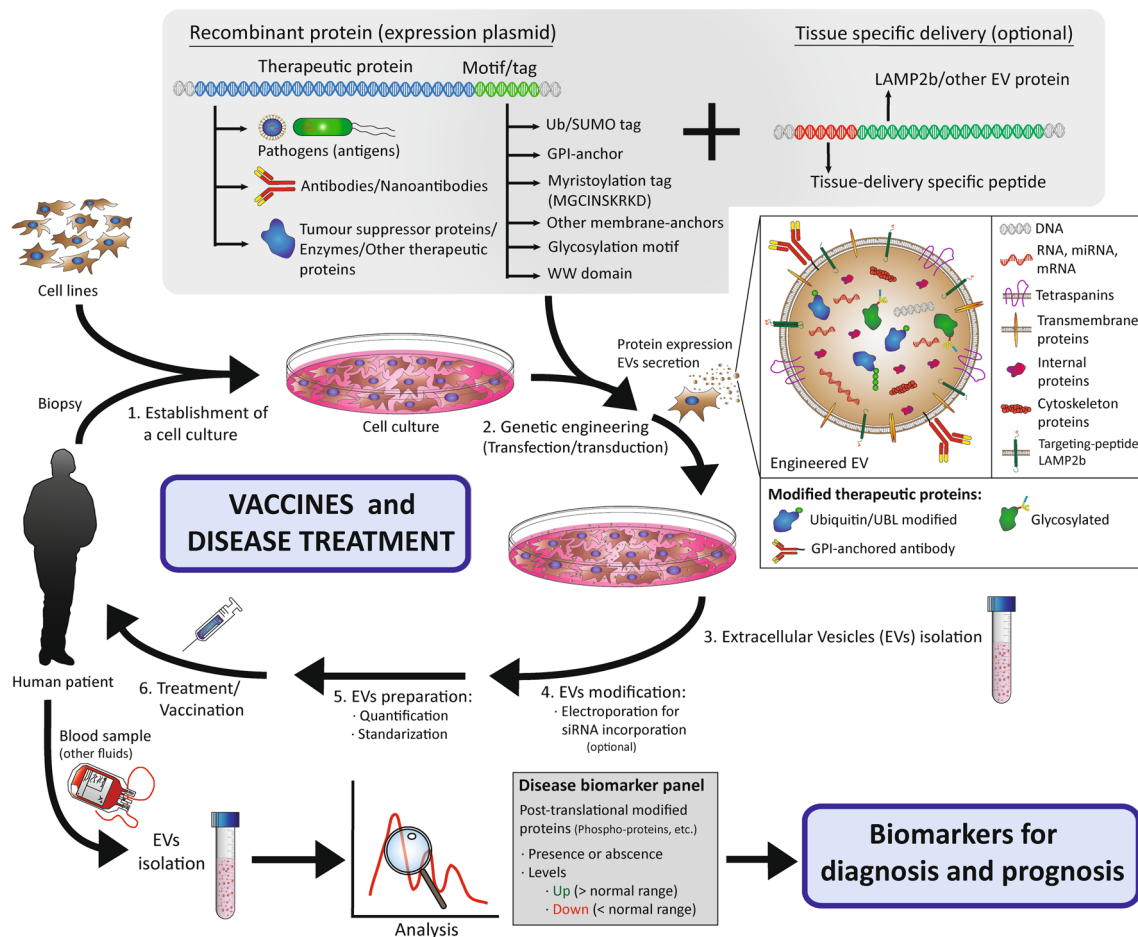
On the other side, the role of the other PTMs, gathered in Table 1, remains largely unexplored. This could suggest that a role for these PTMs is unlikely, or that they can work as a cellular retaining signal for some proteins, directing them to other cellular processes such as the autophagy-lysosome degradation pathway.

## Targeting post-translationally modified proteins into EVs for biomedical applications

Deciphering the role of PTMs for protein sorting into exosomes could be useful for medical purposes, such as association of certain modified proteins with concrete physio-pathological situations, highlighting PTMs of exosomal proteins as biomarkers for prognosis, diagnosis and treatment of diseases.

## PTM marks in EVs as biomarkers for diagnosis and prognosis

EVs have potential as non-invasive biomarkers for several pathological conditions, particularly due to their accessibility from biological fluids, facilitating the detection of certain pathologies [130–133]. A specific PTM of a protein in EVs in particular diseases could constitute a potential biomarker (Fig. 3). For example, the secretion of modified Tau protein aggravates development and progression of neurodegenerative processes. The majority of modified (phospho-Thr-181) Tau detected in human cerebrospinal fluid samples is secreted via exosomes, constituting a well-characterized



**Fig. 3** Targeting post-translationally modified proteins into EVs for biomedical applications. The process for exosome engineering to use as a vaccine or treatment comprises various steps: (1) EVs can be isolated from different cell cultures, either cell lines or cells obtained from human biopsies. (2) These cells can be transfected or transduced with specific constructs coding for engineered therapeutic proteins, that will be targeted to EVs by the addition of a specific PTM motif or tag. Therapeutic proteins include: pathogen antigens for the generation of vaccines; antibodies or nanoantibodies for activation or blocking of signalling pathways, or any kind of protein with a therapeutic potential. Besides, the expression of LAMP2b fused

to a tissue-delivery targeting-peptide defines a specific fate for these EVs once in the blood stream. (3) The EVs can be isolated by diverse procedures. (4) Additionally, an extra step can be performed to incorporate inside EVs therapeutic small interference RNAs by electroporation. (5, 6) Finally, EVs preparations, once standardized and quantified, could be administered intravenously for disease treatment or vaccination. On the other hand, EVs obtained from blood samples (or other fluids) can be analysed to identify certain condition biomarkers (post-translational modified protein) for disease prognosis and diagnosis. The images in the figures are not scaled

biomarker for Alzheimer's disease (AD) [115]. In fact, ph-Tau in combination with A $\beta$  activate diverse signalling pathways, increasing the production of hyperphosphorylated Tau oligomers, which are then secreted by exosomes within the brain, accelerating neurodegeneration [110]. Moreover, some AD-related proteins such as ph-Tau, A $\beta$ 1-42, neurogranin (NRGN) and the repressor element 1-silencing transcription factor (REST) contained in neuronal-derived exosomes appear in plasma [117]. The transference of these proteins contained in exosomes isolated from AD patients' plasma into the central nervous system of a normal mouse causes Tau aggregation and induces AD-like neuropathology [117]. Other studies carried out with Tau-transgenic mice (rTg4510) revealed a high grade of pathology and higher accumulation of Tau in their exosomes compared to control ones [113].

On the other hand, several phosphorylations of IRS-1 in different residues associate to insulin resistance syndrome in diabetes mellitus as well as to degenerative diseases such as AD and fronto-temporal dementia [111]. Exosomes released by retinal pigment epithelial (RPE) cells into vitreous humour contain a vast subset of phosphorylated proteins in age-related macular degeneration (AMD) patients [108]. Oxidative stressed RPE cells modify the phosphorylation profile of exosomal proteins compared to control cells, thus their detection could represent a source of biomarkers for diagnosis and prognosis of eye-degenerative diseases [108].

Besides, detection of modified exosomal proteins bears potential as a biomarker of treatment progression. For instance, cetuximab works as a therapeutic antibody to block EGFR activation in tumour cells [116]. EVs coming from cetuximab-treated cells represent well their originating cells, containing lower level of phosphorylated EGFR after treatment [116]. Monitoring total and phosphorylated forms of EGFR present in EVs from patient blood could reflect the efficacy of anti-tumour therapy [116].

Moreover, detection of some exosomal biomarkers can reveal the existence of side effects during treatment. For example, a fraction of kidney transplant patients treated with tacrolimus as part of the immunosuppressive therapy developed hypertension 6 months after surgery [114]. Urinary exosomes of hypertensive patients are enriched in the phosphorylated form of thiazide-sensitive NCC compared to non-hypertensive patients [114]. Hence, the measurement of total and phosphorylated NCC in urinary exosomes can be used as a biomarker of hypertension [114].

## Vaccines

EVs also represent a promising tool for other therapeutic applications, such as generation of vaccines. EVs and many viruses share common mechanisms for protein sorting and particle release [134]. The study of EVs physiology can

help us to understand virus infections, but also to generate virus-like particles suitable for vaccination exploiting the exosome pathway by sorting virus proteins into exosomes. For instance, exosomes isolated from infected cells can prime mice and protect them from a following lethal infection [135]. In the case of macrophages infected with the intracellular bacteria *Mycobacterium tuberculosis*, more than 40 mycobacterial components appear in exosomes [136] (Table 2). In addition, the administration of soluble mycobacterial proteins in human epithelial and mouse macrophage cell lines promotes sorting of similar bacterial proteins into exosomes [136]. Ubiquitination of mycobacterial proteins such as HspX and GroES acts as a signal for their incorporation into ILVs and secretion as EVs [136]. Moreover, vaccination using these exosomes in mice protects them against subsequent aerosolized infection with the whole infective bacteria [136]. This is just an example of how vaccination with a PTM-protein is a new strategy to initiate a protective immune response against a specific pathogen. An option for heterologous protein sorting into exosomes, which can be used as a vaccine, consists of protein overexpression with the addition of an ubiquitin tag, avoiding strategies based on inactivated or non-replicative pathogens [128, 137] (Fig. 3). This approach enables the exosomal delivery of proteins not usually found in exosomes, e.g. GFP. For instance, the use of exosomes produced by a stable cell line and containing recombinant fusion proteins from *M. tuberculosis* evoked T cell immune responses [137]. However, although exosome-based vaccines could trigger antigen-specific immune responses, they also contain other proteins that could trigger side- or off-target effects. In addition to ubiquitin, GFP SUMOylation drives it into exosomes, suggesting that SUMO could work as a specific sorting tool for antigen incorporation into exosomes for vaccination schemes [106] (Fig. 3).

## Disease treatment

EVs are a promising tool to design new strategies for disease treatment. The size of these small particles make them suitable to carry proteins, lipids, RNA, DNA or small chemical drugs, protecting them from external factors in the blood stream [138, 139]. They can be easily isolated in high amounts from diverse culture cells types. Furthermore, the target specificity of EVs prevents some secondary effects observed in traditional therapies [138]. One of the most potent advantages of exosomes is their capability of crossing the brain blood barrier (BBB), which opens a wide variety of possibilities for diagnosis and treatment of neurodegenerative diseases [140]. In fact, exosomes carrying a specific siRNA and a brain-delivery peptide signal could even silence the expression of the targeted gene [141]. Generating exosomes targeted to a certain type of tissue can be

accomplished by the expression of specific tissue-delivery peptide fused to the N-t of lysosome-associated membrane glycoprotein 2 (LAMP2), a transmembrane protein which causes their incorporation into EVs [141]. Engineered targeting peptides-LAMP2 sometimes undergo degradation even when localized to exosomes, reducing their potential as therapeutic tools. A recent study has discovered that the incorporation of glycosylating motifs enhances their delivery and protects them from damage [142].

The use of engineered EVs based on biologically active proteins could be used to treat some diseases. The addition of a WW tag (a domain-containing PPxY motif) to a certain protein determines its incorporation into exosomes [139]. The L-domains of Ndfip1 recognize this particular mark, targeting this protein to exosomes [14]. In the same way, we propose the use of known PTMs motifs, such as the myristoylation sequence MGCINSKRKD, in the N-t of the protein of interest to drive it specifically into exosomes, as other authors have described for Tya [124]. Moreover, addition of a C-t ubiquitin or SUMO tag could deliver proteins into exosomes, including targeting-peptides-LAMP2 with glycosylated motifs for specific tissue delivery [106, 137, 142]. Besides, protein glycosylation has improved the efficacy of therapeutic proteins [143]. Thus, addition of glycosylating motifs to the protein of interest could also mediate its incorporation to exosomes, although protein glycosylation is still technically complex for industrial production [73, 107] (Fig. 3).

PTM-dependent sorting approaches could contribute to halting tumour progression and development. PTEN, a tumour suppressor phosphatase, is ubiquitinated in exosomes and is active in recipient cells, promoting the dephosphorylation of AKT [78]. Hence, the uptake of exosomes containing the C-t domain of PTEN by cancer cells reduces proliferation, migration and metastasis, postulating such a strategy as an anti-tumour treatment [144]. On the other hand, GPI modification of blocking-nanoantibodies that recognize receptors present in tumour cells, favours their attachment to exosomes. Exosome-attached EGFR-nanoantibodies approach can confer a specific selectivity to EVs against tumour cells, decreasing their EGFR-proliferative signalling pathways [145]. Hence, GPI anchors can be used to target particular proteins to EVs for biomedical applications [146] (Fig. 3). To sum up, all these strategies remark the potential application of EVs-containing post-translational modified proteins for selective delivery of therapeutic components into specific tissues.

## Concluding remarks

Although the mechanisms that define the sorting of proteins into EVs are not fully understood, PTMs are likely to play

an important role. Many post-translationally modified proteins have been detected in EVs [73, 107], whereas others that have not been identified in EVs could have a role in protein delivery, participating in the incorporation of other proteins, or being removed before secretion. Ubiquitination or SUMOylation of some proteins have a clear role in their incorporation into exosomes, whereas others such as ISGylation or acetylation oppose such mechanisms, driving the modified protein to degradation [106, 128, 137]. Moreover, exosome secretion can be mediated by diverse mechanisms, such as the ESCRT complex, lipids and tetraspanins, increasing the complexity and diversity of ILV-protein sorting and exosome release [18]. In this regard, EVs populations are heterogeneous as each vesicle contains a specific repertoire of proteins potentially different from its neighbours [10, 11]. In addition, there are other factors that increase the complexity of this system, for example the fact that almost every cell can secrete EVs containing common and cell-type-specific markers. Then, the final destination of each population of EVs can be different, working as an autocrine or paracrine signal or even travelling throughout the blood stream to other tissues.

Regarding ubiquitination, its own structure and functional complexity increases the number of factors to be taken into consideration. Compartmentalization and specificity of the different E3 ligases participate in protein localization [93, 94]. In addition, competition or crosstalk with other PTMs such as SUMOylation or Neddylation alters the fate of the modified protein [100]. Also, the complexity of the ubiquitin code includes homotypic, heterotypic, mixed or branched chains that distribute protein populations among many cellular processes such as proteasome degradation or endocytic trafficking, forming a ubiquitin-based protein ZIP code that has yet to be revealed [20–25, 33, 147]. Finally, the study of ubiquitinated proteins or even UBLs is difficult technically. Proteomics approaches to find ubiquitinated proteins in whole proteome typically used trypsin digestion of the sample. Nowadays, the discovery of new UBLs highlights that the sole use of this enzyme may mask the appearance of additional variations due to the same di-Gly residue shared by all these kind of modifications [148]. Hence, this information can only be used to localize specific sites on proteins susceptible to be modified by these kind of PTMs [148]. Nevertheless, some approaches tried to discern among these PTMs by terminal mutation and protein overexpression [149]. Besides, these proteomic approaches could also help to discover or define any possible consensus sequence for protein delivery into EVs, either protein mono-ubiquitination and selective chain formation or other PTMs [102, 150, 151].

In addition, EVs represent a novel non-invasive and selective therapeutic tool to be developed for prognosis, diagnosis or treatment of diverse diseases, with very promising



preliminary results [138, 139, 152, 153]. PTMs confer specific properties to proteins, playing a relevant role in EVs protein sorting. Hence, the use of PTMs represents a useful tool for the generation of therapeutically engineered EVs.

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# Post-translational modifications of exosomal proteins

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Exosomes mediate intercellular communication and participate in many cell processes such as cancer progression, immune activation or evasion, and the spread of infection. Exosomes are small vesicles secreted to the extracellular environment through the release of intraluminal vesicles contained in multivesicular bodies (MVBs) upon the fusion of these MVBs with the plasma membrane. The composition of exosomes is not random, suggesting that the incorporation of cargo into them is a regulated process. However, the mechanisms that control the sorting of protein cargo into exosomes are currently elusive. Here, we review the post-translational modifications detected in exosomal proteins, and discuss their possible role in their specific sorting into exosomes.

**Keywords:** post-translational modifications, exosomes, ubiquitination, sorting, multivesicular bodies

## INTRODUCTION

Post-translational modifications (PTMs) of proteins are biochemical changes generated after the synthesis of polypeptides on ribosomes. PTMs include changes to the chemical nature of amino acid residues and also structural modifications that affect the interactive ability of proteins, and consequently their stability, subcellular localization, and activation state (1, 2). There are many types of PTM that can be classified according to the nature of the materials added: (1) a chemical group (phosphate, acetate, etc.), (2) carbohydrates, (3) lipids, (4) amino acids, (5) other polypeptides, and (6) an isoprenyl group (Table 1). A protein can undergo many PTMs, changing its properties and broadening its capacity to adapt to cellular needs (2). Some modifications are reversible and are strictly regulated by the enzymes responsible for their addition or removal, acting as a dynamic switch that allows the cell to adjust protein functions according to requirements. Dysregulation of PTMs or mutation of modified residues are linked to disease, including cancer, neurodegenerative disorders such as Alzheimer, and cardiovascular disease, highlighting the importance of these protein modifications (3–7).

A specific pattern of PTMs is detected in exosomes, 50–200 nm diameter vesicles secreted by most cells to the extracellular environment. Once released, exosomes can adhere to or be internalized by recipient cells, and in this way mediate cell-to-cell communication in a variety of contexts. Exosomes form through the invagination of the limiting membrane of specific endosomal compartments called multivesicular bodies (MVBs) (22). The resulting intraluminal vesicles (ILVs) are released as exosomes upon fusion of MVBs with the plasma membrane. Alternatively, MVBs can fuse with lysosomes, leading to degradation of their content. Exosomes have a specific composition of lipids, proteins, and RNAs; however, the mechanisms that control the sorting of molecules into these exosomal-proteins vesicles remain elusive. Here, we review the PTMs detected in exosomal proteins, and discuss their possible role in their specific sorting into exosomes.

## UBIQUITINATION AND SUMOylation

Post-translational modifications increase the versatility of proteins by influencing their activation state, stability, subcellular localization, and ability to interact with other proteins. A particularly effective means of increasing protein versatility is the addition of ubiquitin, which can be attached to a target protein at a number of positions and in a variety of ways. The C-terminal glycine of ubiquitin usually forms an isopeptide bond with the ε-amino group of a lysine residue present in the target protein, resulting in mono-ubiquitination. In some cases, E4 ubiquitin ligases can add a poly-ubiquitin chain to a mono-ubiquitinated site (23). The equation becomes even more complicated considering that ubiquitin has seven lysines, and the fate of the target protein is determined by which lysine forms the link in the poly-ubiquitin chain: chains linked through lysine-48 (Ub-K48) label target proteins for degradation in the proteasome; Ub-K63 chains seem to be important for the DNA-damage response, endocytosis, autophagy, and signal transduction; Ub-K11 chains are implicated in endoplasmic-reticulum-associated degradation (ERAD); and Ub-K29 chains are involved in lysosomal degradation (24–33). Moreover, in some cases, ubiquitin can be linked through residues other than lysine, such as the N-terminal through the free amino group or the sulfhydryl group of cysteine residues (34). Ubiquitination can also compete with other PTMs, such as sumoylation or acetylation, and can enhance others such as phosphorylation (35–37).

Ubiquitination, thus denotes a complex network of PTMs, and its role in the sorting of proteins into exosomes is far from understood. There seems to be consensus that ubiquitination is necessary for sorting proteins into ILVs destined for degradation through the fusion of the encompassing MVB with lysosomes. This process is mediated by the endosomal sorting complex required for transport machinery (ESCRT complex) and affects proteins such as epithelial growth factor receptor (EGFR) (38) (Figure 1). This machinery recognizes ubiquitinated cargoes and catalyzes the abscission of endosomal invaginations, forming ILVs that contain the sorted

Table 1 | Post-translational modification of eukaryotic proteins.

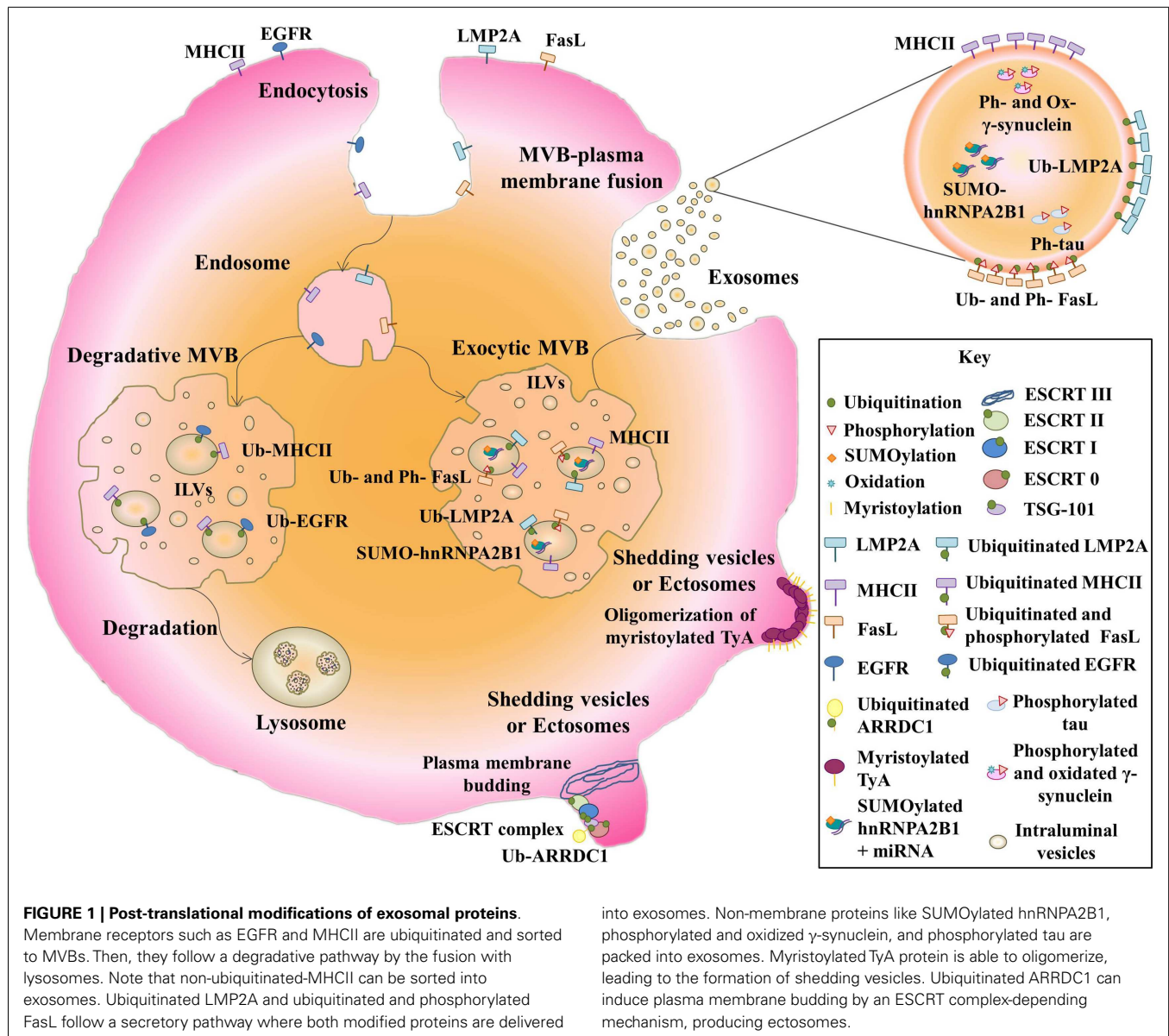
Groups of PTMs	Modification	Added group	Modified residues of target proteins	PTM in exosomal proteins	Biological relevance conferred by the PTMs	Reference
<b>POST-TRANSLATIONAL MODIFICATIONS OF EUKARYOTIC PROTEINS</b>						
Addition of a chemical group	Phosphorylation	Phosphate group	Tyr, Thr, Ser, His	FasL; AnnexinA2; tau; $\gamma$ -synuclein	Sorting into exosomes; incorporation into exosomal membrane; spreading of toxic aggregates through exosomes; spreading of toxic aggregates through exosomes	(8–11)
	Acetylation	Acetyl group	Lys	–	–	–
	Methylation	Methyl group	Lys, Arg	–	–	–
	Oxidation	Different oxygen species	All amino acids, but preferentially Tyr, Phe, Trp, His, Met, Cys	$\gamma$ -Synuclein	Spreading of toxic aggregates through exosomes	(11)
	Nitrosylation	Nitric oxide (NO)	Cys, Met	–	–	–
Addition of carbohydrates or glycosylation	N-linked glycosylation	Glycosyl group	Asn, Arg, and N-terminus	Several glycoproteins	Sorting of particular glycoproteins into exosomes (?)	(12–14)
	O-linked glycosylation	Glycosyl group	Ser, Thr, and amino acids in close proximity to Tyr phosphorylation sites	Several glycoproteins	Sorting of particular glycoproteins into exosomes (?)	(12–14)
	C-linked mannosylation and glypiation [glycosylphosphatidylinositol (GPI) anchor]	Glycosylphosphatidylinositol (GPI) group	Carbon on a tryptophan side-chain and C-terminus, respectively	Several glycoproteins	Sorting of particular glycoproteins into exosomes (?)	(12, 13)
Addition of lipids (lipidation)	Palmitoylation	Palmitic acid	Cys	–	–	–
	N-myristoylation	Myristoyl group	N-terminal glycine residue	Artificial conjugation of TyA protein to myristoyl group	Sorting of TyA into shedding vesicles	(15)
Addition of amino acids	Polyglutamylation	Glutamic acid	Gly	–	–	–

(Continued)

Table 1 | Continued

Groups of PTMs	Modification	Added group	Modified residues of target proteins	PTM in exosomal proteins	Biological relevance conferred by the PTMs	Reference
Addition of other polypeptides	Ubiquitination	Ubiquitin protein	Lys, N-terminus, non-lysine residues (Cys, Thr, Ser)	Several proteins, some examples: LMP2A; PTEN; SIMPLE; HSP70; ARRDC1	Exosomal LMP2A loading; Sorting of PTEN into exosomes; Unknown function; Secretion into shedding vesicles	(16–20)
	SUMOylation (Small ubiquitin-related modifier addition)	SUMO1-4 proteins	Tetrapeptide consensus motif $\Psi$ -K-x-D/E ( $\Psi$ : hydrophobic residue, K: lysine conjugated to SUMO, x: any amino acid, D or E: acidic residue)	hnRNPA2B1	Regulate the binding of miRNAs to hnRNPA2B1	(21)
	NEDDylation (neural-precursor-cell-expressed developmentally down-regulated 8 addition)	NEDD8 protein	Lys	–	–	–
Isoprenylation	ISGylation (Interferon-stimulated gene 15 addition)	ISG15 protein	Lys	–	–	–
	Farnesylation	Farnesyl group	Cys and sequence motifs CAAX, CC, or CAC at C-terminus (C: Cys, A: alanine, X: any amino acid)	–	–	–
	Geranylgeranylation	Geranylgeranyl isoprene unit	Cys and sequence motifs CAAX, CC or CAC at C-terminus (C: Cys, A: alanine, X: any amino acid)	–	–	–





cargo [reviewed in Raiborg and Stenmark (39)]. The ESCRT complex consists of four subcomplexes, ESCRT-0, -I, -II, and -III, and several accessory proteins. ESCRT-0, -I, and -II contain ubiquitin-binding subunits that interact directly with ubiquitinated cargo. The directional flow of cargo from ESCRT-0 to ESCRT-I and -II might be regulated by PTMs. In fact, the ESCRT-0 subunits are known to be phosphorylated and to be mono-ubiquitinated (40–42). The latter modification keeps these subunits in an inactive form owing to intramolecular interactions between their ubiquitin interacting motifs and the appended ubiquitin (43, 44). However, the role of ubiquitin and the ESCRT complex in the sorting of proteins into ILVs for exosome secretion is still unclear, and MVB biogenesis, exosome secretion, and exosomal-protein sorting have been reported in an ESCRT/ubiquitin-independent manner [reviewed at Villarroya-Beltri et al. (45)].

The Epstein-Barr virus (EBV) protein LMP2A (latent membrane protein 2A) is ubiquitinated in exosomal fractions (16). LMP2A contains two PXY motifs, through which it associates with neural precursor cell expressed developmentally down-regulated protein 4 (Nedd4)-family ubiquitin ligases (16). Ubiquitination of LMP2A leads to endocytic transport of the protein from the plasma membrane to MVBs. Nedd4 E3 ubiquitin ligases are able to bind directly to target proteins through the PPXY motif, but proteins lacking this motif can bind Nedd4 through the adaptor Nedd4-family-interacting protein 1 (Ndfip1), leading to their ubiquitination (46). Ndfip1 is involved not only in protein degradation, but also in protein traffic to exosomes (46). Ndfip1 overexpression increases the protein content of exosomes and enhances exosomal sorting of normally absent proteins, such as Nedd4, Nedd4-2, and Itch. These exosomal proteins moreover



appear to be highly ubiquitinated, suggesting that Ndfip1 transfers other ubiquitinated proteins to exosomes (46) (**Figure 1**). For example, the Ndfip1 adaptor function is required for exosomal export of phosphatase and tensin homolog deleted on chromosome 10 protein (PTEN) ubiquitinated on lysine 13 (17).

With other proteins, however, ubiquitination appears to be unimportant or inhibitory to exosomal export. Secretion into exosomes of small integral membrane protein of the lysosome/late endosome (SIMPLE) is enhanced by mutations in its PPXY motif, which mediates its binding to E3 ubiquitin ligases (18). Ubiquitination is also not required for the packaging of major histocompatibility complex II (MHC-II) into exosomes (47). The use of a chimeric-ubiquitinated-MHC-II molecule does not specifically lead these molecules into exosomes, and forcing MHC-II ubiquitination by expression of membrane-associated ring finger (C3HC4) 8 (MARCH) E3 ubiquitin ligase does not enrich MHC-II molecules in exosomes, though it does completely deplete them from the plasma membrane. Moreover, directed-mutagenesis of all MHC-II lysine residues does not impair the exosome sorting of these receptors (47). However, ubiquitination of the MHC-II cytoplasmic domain, required for recognition by the ESCRT complex, is important for sorting membrane MHC-II to MVBs for lysosomal degradation (48). The non-ubiquitination of MHC-II molecules present in exosomes suggests that this PTM is not involved in sorting to these vesicles. The two mechanisms for loading MHC-II into MVBs are engaged for different physiological functions. Thus, whereas ubiquitin-dependent sorting takes place in immature DCs, in which ubiquitinated receptors are degraded in lysosomes, in activated-DCs, non-ubiquitinated MHC-II-containing exosomes are efficiently delivered to interact with T cells, enhancing antigen specific MHC-II-mediated presentation (48) (**Figure 1**). Heat shock protein 70 (HSP70) also seems to be sorted into exosomes independently of its ubiquitination. Thus, although deletion of the deubiquitin domain of COP9 signalosome complex subunit 5 (CSN5) enhances packing of ubiquitinated HSP70 into exosomes, knockdown of the entire CSN5 protein increases the levels of both modified and non-modified HSP70 in exosomes (19).

Mass spectrometry analysis of PTMs in extracellular vesicles released by insulinoma cells identified multiple poly-ubiquitinated proteins (49). Enrichment of exosomes in poly-ubiquitinated proteins was also demonstrated by an approach based on the use of FK1 antibody (which only binds poly-ubiquitinated proteins) and P4D1 (which labels poly- and mono-ubiquitinated proteins) (50). Other studies suggest that exosomal proteins are preferentially mono-ubiquitinated or de-ubiquitinated, based on western analysis showing discrete ubiquitinated protein bands rather than smeared bands (19).

Ubiquitination has been shown to be important for the secretion of a novel type of extracellular vesicle, distinct from exosomes, called arrestin-domain-containing protein 1 (ARRDC1)-mediated microvesicles (ARMMs). ARMMs directly bud from the plasma membrane upon interaction of the tumor susceptibility gene 101 protein (TSG101) with a PSAP motif in ARRDC1, which is localized through its arrestin domain at the plasma membrane (20). ARRDC1 in vesicles is ubiquitinated by the E3 ligase WW domain-containing protein 2 (WWP2). Down-regulation of

WWP2 decreases ARRDC1 protein level in vesicles, and a PPXY-mutant of ARRDC1 strongly inhibits ARMM secretion, suggesting that ARRDC1 ubiquitination promotes ARRDC1 sorting into vesicles and ARMM secretion (20) (**Figure 1**).

Another ubiquitin-like modifier called small ubiquitin-related modifier (SUMO) has been found to modify the exosomal-protein heterogeneous nuclear ribonucleoprotein A2B1 (hnRNPA2B1). This modification affects the ability of this protein to export micro ribonucleic acids (miRNAs) into exosomes, probably by affecting its binding to miRNAs (21). hnRNPA1 in exosomes was also found to be modified, increasing its molecular weight by about 12 kDa on gel electrophoresis (21) (**Figure 1**). This change in molecular weight of hnRNPA2B1 and other proteins has been shown before (49).

## OTHER PTMs: PHOSPHORYLATION AND GLYCOSYLATION

Mass spectrometry analysis of extracellular vesicles also detects phosphorylated proteins (49). Phosphorylation and ubiquitination co-regulate sorting of Fas ligand (FasL) into secretory lysosomes by controlling its entry into MVBs (8). FasL contains a proline-rich domain (PRD) in the cytosolic tail to which tyrosine kinases, such as FGR, FYN, and LYN bind, and phosphorylation of tyrosine residues by these kinases enhances internalization to MVBs. The flanking regions of the PRD contain lysines, which are mono-ubiquitinated. Mutation of these lysines impairs the localization of FasL in MVBs, but mutation of the tyrosines does not affect mono-ubiquitination. Phosphorylation is thus not required for ubiquitination, but both PTMs are necessary for incorporation of FasL into to MVBs (8) (**Figure 1**).

Phosphorylation is also involved in incorporation of the  $\text{Ca}^{2+}$ -dependent phospholipid-binding protein Annexin A2 into exosomal membranes, through the action of raft-resident kinases, such as SRC or LYN on Tyr-23 (9). Aberrant phosphorylation of the protein tau on threonine-181 promotes its incorporation into exosomes, resulting in the spreading of this abnormally processed protein in Alzheimer disease patients (10).

The protein  $\gamma$ -synuclein is transported in exosomes in its modified form. This modification consists of oxidation of Met-38 and Tyr-39, which confers prion-like properties and causes the formation of toxic aggregates. The spreading of these aggregates is in part mediated by the exosomal transport of oxidated- $\gamma$ -synuclein to glial cells (11) (**Figure 1**).

Carbohydrate modifications, involved in protein trafficking, cellular recognition, and communication of cells with their extracellular environment, have also been studied in extracellular vesicles (12, 13, 51). Vesicles of diverse cells types are enriched in proteins with high mannose, polylectosamine,  $\alpha$ -2,6-sialic acid, and complex N-linked glycans adjuncts; in contrast, there is a comparative under-representation of specific glycan epitopes, such as terminal blood group A and B antigens (12, 13). Exosome glycan profiles of different cell sources, such as T-cells, melanoma and colon cancer cells, and biological fluids like breast milk, are very similar, although they conserve some features of their parent membranes (13). The carbohydrate fingerprint detected in exosomes is less diverse than that observed in parent cells, but correspond to a conserved fraction of the parent cellular membrane that display a particular glycan profile (13). The variability observed between

cellular and exosomal carbohydrate signatures has been suggested to indicate different membrane microdomain origins of these vesicles (13). It has been described that polyLacNAc and high mannose modifications associated with galectins and VIP36 are responsible for the oligomerization of glycoproteins that mediate their sorting into Golgi-derived vesicles (12, 52–55). Galectins and galectin-associated proteins have also been detected in exosomes so it is possible that glycosylation may also play a role in the sorting cargo into extracellular vesicles (12, 13).

Membrane anchors have also been shown to be important for the budding of vesicles derived from the plasma membrane. An N-terminal acylation tag serves as a signal for the import of highly oligomeric cytoplasmic proteins, like the yeast protein TyA, into shedding vesicles (56). The membrane anchor that most effectively promotes TyA budding is myristoylation. However, targeting of TyA to the endosomal membrane by fusion to a Phosphatidylinositol-3-phosphate (PI3P) binding domain does not produce the same effect (15) (**Figure 1**).

## CONCLUDING REMARKS

Post-translational modifications decorate proteins and drive their fate in cells by affecting multiple parameters including stability and localization. Different modifications can affect the same protein; sometimes competing with each other or being mutually exclusive, but in other cases can promote other modifications. Enzymes controlling PTMs additionally show very specific patterns of expression, activation, and subcellular localization, exponentially increasing the diversity and potentiality of cellular proteomes.

Different types of PTMs have been found in exosomal proteins; however, the role of these modifications in the localization of proteins into exosomes is not clear. The enigmatic role of ubiquitination, whose final consequences seem to differ depending on the target protein, is a particular case in point. The type of ubiquitination may also account for the fate of the modified protein, and could be a key determinant for its loading in exosomes. In some cases, ubiquitination seems to target the protein into MVBs destined for degradation, whereas sorting of proteins into MVBs that fuse with the plasma membrane to release exosomes seems to be ubiquitin-independent, clearly pointing to the existence of different types of MVB with different sorting mechanisms.

Specific protein modifications can reflect a particular pathological condition. The presence of modified proteins in exosomes can therefore make them invaluable tools for diagnosis, since modifications could be easily detected in exosomes obtained from body fluids without the need for invasive tissue biopsies.

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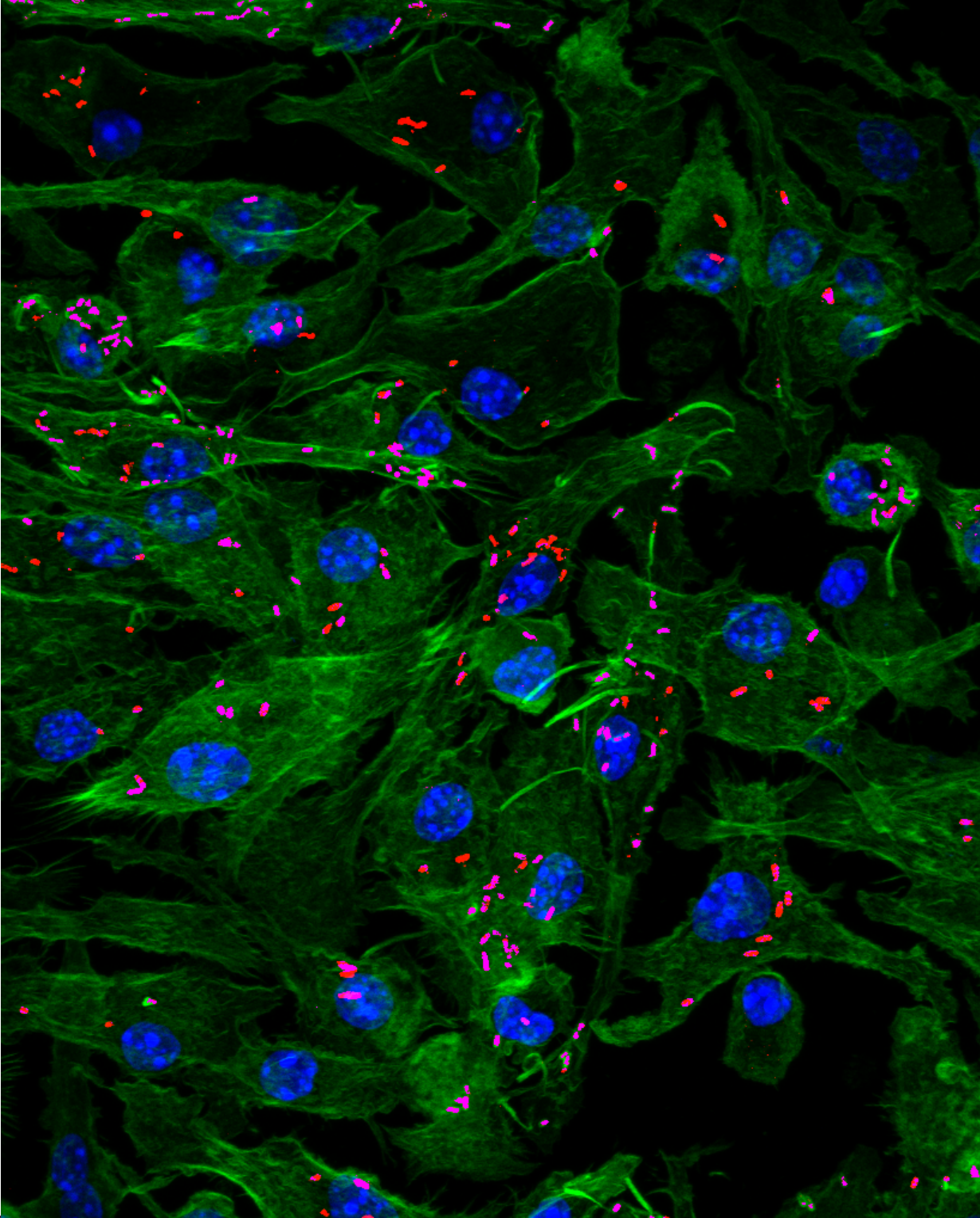
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